VNIVERSITAT DE VALÈNCIA Facultat de Farmàcia

Doctorado en Biomedicina y Farmacia

Vesículas extracelulares derivadas de células madre mesenquimales de tejido adiposo como terapia biológica en células articulares osteoartríticas

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Valencia, 2018

DEPARTAMENT DE FARMACOLOGIA

María José Alcaraz Tormo, Catedrática de la Universitat de València y María **Isabel Guillén Salazar**, Profesora Titular de la Universidad CEU-Cardenal- Herrera.

CERTIFICAN:

Que el trabajo presentado por el Licenciado Miguel Tofiño Vian, titulado: "Vesículas extracelulares derivadas de células madre mesenquimales de tejido adiposo como terapia biológica en células articulares osteoartríticas" ha sido realizado en el *Departament de Farmacologia* de la *Universitat de València*, bajo nuestra dirección y asesoramiento.

Concluido el trabajo experimental y bibliográfico, autorizamos la presentación de esta Tesis Doctoral para que sea juzgada por el Tribunal correspondiente.

Valencia, a 10 de Octubre de 2018

María José Alcaraz Tormo

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La presente tesis doctoral ha sido financiada gracias a las siguientes ayudas:

Proyecto SAF2013-4874R concedido y financiado por el Ministerio de Economía y Competitividad, bajo el título: “Mecanismos celulares reguladores de la respuesta inflamatoria en patologías inflamatorias crónicas”.

Proyecto PROMETEOII/2014/071 a Grupos de Investigación de Excelencia concedido y financiado por la Generalitat Valenciana bajo el título: “Estrategias de protección frente a procesos inflamatorios y degenerativos”.

Proyecto RETICEF RD12/0043/0013 concedido y financiado por el Instituto de Salud Carlos III, bajo el título: “Red de Investigación en envejecimiento y fragilidad”.

Contrato Predoctoral PROMETEO para Personal de Investigación en Formación, concedido y financiado por la Generalitat Valenciana.

Para Aida

Érase una vez un pobre iluso que concluyó una tesis doctoral. Cuando llegó el momento de echar la vista atrás y contemplar el camino recorrido, tomó aire y tragó saliva. Había tanta, tantísima gente a quien agradecer; tantas personas sin las que no habría habido camino. Espero que estas palabras sirvan, aunque sea un poquito, para reconocer el apoyo y la generosidad que he recibido.

Agradezco a mis directoras: María José Alcaraz, por tu apoyo constante y tu sabiduría, con la que tanto he aprendido; e Isabel Guillén, por tu pasión, tus consejos, tus burlas y tus conversaciones interminables, cada una más enriquecedora que la anterior —y alguna, incluso, sobre ciencia.

A los profesores de Farmacología, que han compartido su casa conmigo durante estos años. En especial, a Maricarmen Terencio, por tu cajón infinito de consejos y ayuda, por tu sonrisa perenne cada mañana al entrar al laboratorio. A todo personal de secretaría y, entre ellos, a Mati y Mamen.

A la profesora Edit Buzás, que con su amabilidad e inteligencia convirtió mi estancia en Hungría en una de las mejores experiencias que recuerdo. A mis compañeros húngaros: Árpád, Tamás, Orsolya, Eszter, Anita y especialmente András, por nuestra amistad y nuestros paseos nocturnos en Budapest y Barcelona —y todos los que vendrán.

A mis compañeros y amigos del laboratorio. Julia, la dragona, por haberme enseñado tanto siempre entre risas. Carmen, por ofrecerme siempre tu tiempo. Asun, por preguntarme a cada rato por lo que escribo; Laura, por tu saber hacer y nuestra retroalimentación con los *Western blots*; y Josep, por tu buen humor invencible, tus listas de Spotify y tus novelas de romanos. A los alumnos que he tenido la suerte de acompañar: Raúl, con quien compartí tantas historias, y María José, cuya ayuda ha sido tan inestimable como nuestras charlas. A Nuria y Laura por sus reservas infinitas de energía.

Gracias también a mis compañeros del grupo de enfermedades cardiovasculares: Cristina, Fermín, Fran y especialmente Andrea, con tu almacén interminable de chocolate ecuatoriano. Finalmente a María, a quien tengo tanto que agradecer que no sabría por dónde empezar. Gracias por tu bondad, tus buenos consejos, tu apoyo y todas las risas que hemos compartido. Gracias por ser diferente, por darme esperanza y por estar siempre ahí.

Gracias a mis amigos. En especial, a Laia. A Libertad, a quien he descubierto durante estos años. Gracias a mis padres, Miguel y Maricarmen, y mis hermanos Carlos y Javier. Por muy lejos que esté os sigo queriendo con locura.

Por último, gracias a Aida, mi soñadora y mi compañera de travesía. Esta tesis es tan tuya como mía: un pasito en nuestro viaje. Gracias por tanto.

ABREVIATURAS

Alix	Proteína de interacción con muerte celular programada 6
AP	Proteína activadora
ARF	*Ras-related GTPase ADP-ribosylation factor*
ASC	Célula madre mesenquimal derivada de tejido adiposo
CCL	Quimiocina de tipo C-ligando
CD	Clúster de Diferenciación
CM	Medio acondicionado
COX	Ciclooxigenasa
$cPLA_2$	Fosfolipasa A_2 citosólica
CPN	Carboxipeptidasa N
DC	Célula dendrítica
DMEM/HAM F12	Medio de Eagle modificado de Dulbecco / Mezcla 12 de Ham
ELISA	Ensayo de inmunoabsorción ligado a enzima
EM	Microscopía electrónica
ERK	Quinasa regulada por señal extracelular
ESCRT	Complejo endosomal de clasificación requerido para el transporte
EV	Vesícula extracelular
EX	Exosoma
FC	Citometría de flujo
FGF	Factor de crecimiento de fibroblastos
FPR	Receptor de péptido formilado
HGF	Factor de crecimiento hepático
HLA-DR	*Human Leukocyte Antigen – Antigen D related*
Hsc70	Proteína 8 70kDa de estrés térmico
ICAM	Molécula de adhesión intercelular
IFN	Interferón
IL	Interleucina
iNOS	Sintasa de óxido nítrico inducible
ISEV	Sociedad Internacional de Vesículas Extracelulares
LAMP	Proteína de membrana asociada a lisosoma
MAPK	Proteín-quinasa activada por mitógeno

M-CSF	Factor estimulante de colonia de macrófagos
MHC	Complejo mayor de histocompatibilidad
miRNA	Ácido ribonucleico monocatenario *micro*
MMP	Metaloproteinasa de matriz
mPGES	Prostaglandina E sintasa microsomal
mRNA	Ácido ribonucleico mensajero
MSC	Célula madre mesenquimal
MV	Microvesícula
MVB	Cuerpo multivesicular
NF-κB	Factor nuclear-κB
NK	*Natural killer*
NO	Óxido nítrico
NTA	Análisis de rastreo de nanopartículas
OA	Osteoartritis / Artrosis
PBS	Tampón fosfato salino
PCR	Reacción en cadena de la polimerasa
PEG	Polietilenglicol
PGE_2	Prostaglandina E_2
PZP	Proteína de zona de gestación
Rab	Proteína relacionada con Ras en cerebro
RANKL	Ligando de receptor activador para el factor nuclear κB
ROS	Especies reactivas de oxígeno
SEC	Cromatografía de exclusión molecular
TGF-β	Factor de crecimiento transformante-β
TNFα	Factor de necrosis tumoral α
TRPS	Detección de pulso resistivo ajustable
Tsg101	Gen de susceptibilidad a tumores 101
VAMP	Proteína de membrana asociada a vesícula
VCAM	Molécula de adhesión celular vascular
VEGF	Factor de crecimiento vascular endotelial
γH2AX	Histona H2A fosforilada en serina 129

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RESUMEN / *ABSTRACT*

La osteoartritis o artrosis (OA) es la enfermedad de las articulaciones con mayor prevalencia a edades avanzadas y está asociada con una pérdida progresiva del cartílago articular, una sinovitis de bajo grado y una serie de alteraciones deletéreas en el hueso subcondral y los tejidos periarticulares. Con el transcurso de la patología se produce un desequilibrio entre los procesos anabólicos y catabólicos de la articulación que se ven agravados por la presencia de estrés mecánico y una progresiva acumulación de mediadores inflamatorios. Actualmente no existe un tratamiento efectivo para la OA, si bien se están investigando nuevas terapias que detengan la progresión de la enfermedad o minimicen el daño tisular. Entre ellas destacan las células madre mesenquimales (MSCs) o los productos que éstas liberan al espacio extracelular (secretoma). En la presente tesis doctoral hemos estudiado el potencial de las vesículas extracelulares (EVs) presentes en el secretoma de las MSCs derivadas de tejido adiposo (ASCs) como posible terapia biológica heteróloga libre de células en diferentes tipos celulares presentes en la articulación osteoartrítica.

Las MSCs han mostrado amplias propiedades inmunomoduladoras y regenerativas en distintos modelos patológicos. Estas facultades se han atribuido, por una parte, a su capacidad de diferenciación multipotencial, pero también a su actividad paracrina. Se sabe que las MSCs liberan un conjunto heterogéneo de mediadores solubles y EVs cuya interacción con otras células y tejidos podría explicar los efectos antiinflamatorios y anticatabólicos de las MSCs. Desde un punto de vista clínico, el uso del secretoma como terapia presenta ciertas ventajas, tales como una menor inmunogenicidad, una mayor predictibilidad de los resultados o una administración más sencilla y controlable.

Las EVs son secretadas activamente por prácticamente todos los tipos celulares y representan un mecanismo de comunicación intercelular en condiciones fisiológicas y patológicas. Sin embargo, su investigación se ha encontrado con diversos obstáculos por la ausencia de una nomenclatura unificada y la dificultad en la estandarización de los protocolos de caracterización física y bioquímica.

En nuestro trabajo hemos caracterizado dos subtipos vesiculares presentes en el medio acondicionado (CM) de ASCs de distinto tamaño y composición, definidos según la literatura como microvesículas (MVs) y exosomas (EXs). Se ha estudiado su composición proteica por técnicas de espectrometría de masas y microscopía confocal, identificando varias proteínas con potenciales efectos inmunomoduladores y protectores como anexina A1 o peroxirredoxina 6.

La presencia de un estado inflamatorio crónico explica muchas de las alteraciones degradativas que tienen lugar en las articulaciones osteoartríticas. Por esta razón, se han estudiado los posibles efectos inmunomoduladores de las MVs y los EXs en cultivos primarios de condrocitos osteoartríticos y explantes de cartílago estimulados con IL-1β, comparativamente con los efectos previamente descritos del CM de las ASCs. Nuestros resultados indican que ambos tipos de EVs no son tóxicas para las células en las concentraciones de estudio y que reproducen o mejoran los efectos del CM del que proceden. Tanto las MVs como los EXs fueron capaces de reducir la liberación de las citocinas proinflamatorias IL-6 y TNFα y de promover la síntesis de IL-10, una citocina antiinflamatoria. Además, inhibieron la producción de PGE_2 y NO, la actividad enzimática MMP y la transcripción de COX-2 y mPGES-1, así como la activación de los factores de transcripción NF-κB y AP-1. Por otra parte, el tratamiento con EVs incrementó la expresión extracelular de colágeno de tipo II, un marcador de condrocito articular. El bloqueo de anexina A1 en las EVs con anticuerpos específicos revertió la inhibición de la síntesis de IL-6 y la producción de colágeno de tipo II, lo que parece indicar que la anexina A1 interviene en la regulación de ambas moléculas.

El envejecimiento y la exposición crónica a mediadores proinflamatorios y diferentes tipos de estrés pueden derivar hacia una senescencia celular. Así, la acumulación de estrés oxidativo, la ralentización de la actividad celular y la secreción de mediadores inflamatorios contribuyen a la degeneración del tejido articular. En este trabajo hemos evaluado los posibles efectos antiinflamatorios y antisenescentes de las EVs en cultivos primarios de osteoblastos osteoartríticos estimulados con IL-1β. Hemos encontrado que las EVs promueven la liberación de IL-10 e inhiben la producción de IL-6 y PGE_2, pero no la de TNFα. Inhiben la peroxidación lipídica, restauran el potencial mitocondrial de membrana de las células no estimuladas, y reducen la actividad β-galactosidasa asociada a senescencia y la acumulación de focos de histona γH2AX.

Nuestros resultados indican que las EVs constituyen un mecanismo relevante de señalización en el conjunto del secretoma de las ASCs, por lo que pueden representar una nueva estrategia terapéutica para el tratamiento de enfermedades inflamatorias crónicas de la articulación.

Palabras clave: artrosis, inflamación, senescencia, célula madre mesenquimal de tejido adiposo, medio acondicionado, vesícula extracelular, condrocito, osteoblasto.

Osteoarthritis (OA) is the most prevalent joint disease in the elderly and it is associated with a progressive loss of articular cartilage, low-grade synovitis and several deleterious alterations in subchondral bone and periarticular tissues. During the course of the pathology there is an imbalance between joint anabolic and catabolic processes that is aggravated by mechanical stress and the progressive accumulation of inflammatory mediators. Currently, there is no effective treatment for OA, although new therapies that stop the progression of the disease or repair tissue damage have been investigated. These new therapies include the use of mesenchymal stem cells (MSCs) or the products that they release into the extracellular space (secretome). In this thesis we have studied the potential use of extracellular vesicles (EVs) from the secretome of adipose tissue-derived MSCs (ASCs) as a possible heterologous cell-free biological therapy for the treatment of several osteoarthritic joint cell types.

MSCs have shown extensive immunomodulatory and regenerative properties in different pathological models. These capacities have been attributed, first, to their multipotential differentiation ability, but also to their paracrine activity. It is known that MSCs release a heterogeneous set of soluble mediators and EVs whose interactions with other cells and tissues could explain the anti-inflammatory and anti-catabolic effects of MSCs. From a clinical perspective, the use of secretomes as therapy presents certain advantages such as a lower immunogenicity, a greater outcome predictability and a simpler and more controllable administration.

EVs are actively secreted by practically all cell types and represent a novel mechanism of intercellular communication both under physiological and pathological conditions. However, their research has been hampered by several obstacles such as the absence of a unified nomenclature and the difficulty in standardizing physical and biochemical characterization protocols.

In our work, we have successfully characterized two specific vesicular subtypes in the conditioned medium (CM) of ASCs, of different sizes and compositions, defined according to the literature as microvesicles (MVs) and exosomes (EXs). Their protein composition has been studied by mass spectrometry and confocal microscopy, identifying several proteins with potential immunomodulatory and protective effects such as annexin A1 or peroxiredoxin 6.

Chronic inflammation explains many degradative alterations in OA joints. For this reason, the possible immunomodulatory effects of MVs and EXs have been studied in primary cultures of osteoarthritic chondrocytes and cartilage explants stimulated with IL-1β, in comparison with the previously described effects of ASC-CM. Our results indicate that both types of EVs are not toxic for the cells in the studied concentrations and that they replicate or improve the effects of the CM from which they come. Both MVs and EXs were able to reduce the release of proinflammatory cytokines such as IL-6 and TNFα and promote the synthesis of IL-10, an anti-inflammatory mediator. In addition, they inhibited the production of PGE_2 and NO, the enzymatic activity of MMP and the transcription of COX-2 and mPGES-1, as well as the activation of the transcription factors NF-κB and AP-1. On the other hand, the treatment with EVs promoted the extracellular expression of collagen type II, a marker of articular chondrocyte phenotype. EV-Annexin A1 blockade with specific antibodies reversed the inhibition of IL-6 synthesis and the production of collagen type II.

Aging and chronic exposure to proinflammatory mediators and different types of stress can lead to cellular senescence. The accumulation of oxidative stress, the slowing of cellular activity and the secretion of inflammatory mediators contribute to the degeneration of joint tissues in OA. This work has evaluated the possible anti-inflammatory and anti-senescent effects of EVs in primary cultures of osteoarthritic osteoblasts stimulated with IL-1β. First, we have found that EVs promote the release of IL-10 and inhibit the production of IL-6 and PGE_2, but not TNFα. Also, they inhibit lipid peroxidation, restore a mitochondrial membrane potential similar to unstimulated cells, and reduce senescence-associated β-galactosidase activity and the accumulation of histone γH2AX foci.

Our results indicate that EVs are relevant actors in the ASC secretome, and represent a new therapeutic strategy for the treatment of chronic inflammatory joint diseases.

Key words: osteoarthritis, inflammation, senescence, adipose tissue mesenchymal stem cell, conditioned medium, extracellular vesicle, chondrocyte, osteoblast.

INTRODUCCIÓN

CÉLULAS MADRES MESENQUIMALES DE TEJIDO ADIPOSO

1.1 Características

Las células madre mesenquimales (MSCs) son células madre adultas con capacidad auto-regenerativa que pueden diferenciarse hacia varios linajes celulares mesenquimatosos. La Sociedad Internacional de Terapia Celular las define como células con un fenotipo específico —positivas para los Clúster de Diferenciación (CD)73, CD90 y CD105; y negativas para CD34, CD45 y HLA-DR (*Human Leukocyte Antigen – Antigen D Related*)—, crecimiento adherente al plástico *in vitro* y pluripotencia (Dominici, Le Blanc et al. 2006). Las MSCs se aislaron originalmente de la médula ósea como precursores de elementos del estroma; no obstante, durante los últimos años se han obtenido poblaciones similares a MSCs de una amplia gama de tejidos adultos: hueso trabecular y cortical, membranas sinoviales, músculo esquelético, tejido adiposo, sangre periférica y tendón, entre otros (Nery, Nascimento et al. 2013). Teniendo en cuenta sus características regenerativas e inmunomoduladoras, su capacidad de diferenciarse a tipos celulares tales como condrocitos, osteoblastos, adipocitos y miocitos, y su relativa facilidad de cultivo, las MSCs han sido postuladas como potenciales agentes terapéuticos en distintas condiciones patológicas (Jevotovsky, Alfonso et al. 2018).

El aumento del número de orígenes de las MSCs ha traído consigo la necesidad de una definición más estrecha y precisa de las mismas. La primera guía para su caracterización, publicada por la Sociedad Internacional de Terapia Celular en 2006, ha ido quedando obsoleta a la luz de las nuevas y más profundas caracterizaciones de las distintas poblaciones de MSCs (Le Blanc y Davies 2018). Cada vez parece más evidente que estas células provienen de distintos orígenes embrionarios y, aunque similares en sus perfiles fenotípicos, muestran diferencias funcionales y atributos propios de su tejido de origen, incluso tras cultivos *in vitro* prolongados (Reinisch, Etchart et al. 2015). Por ejemplo, diferentes laboratorios han empleado marcadores de superficie celular para caracterizar sus poblaciones de MSCs. Sin embargo, muchos de los marcadores positivos para MSCs también lo son para fibroblastos y otras células madre (Lv, Tuan et al. 2014). Por tanto, no se recomienda la identificación de MSCs exclusivamente a través del patrón de marcadores de superficie que, además de ser variable según el tejido de origen, se altera notablemente durante la extracción de la célula de su nicho *in vivo* y su posterior cultivo en placa (Le Blanc y Davies 2018).

Debido principalmente a su fácil acceso, las MSCs más estudiadas han sido las derivadas de médula ósea y las derivadas de tejido adiposo (ASCs). Las ASCs fueron aisladas por primera vez por Zuk y colaboradores en 2001 (Zuk, Zhu et al. 2001). En los últimos años, se han convertido en una alternativa a las MSCs de médula ósea debido a su capacidad proliferativa *in vitro* y a que su protocolo de obtención es más fácil, menos invasivo y más seguro, alcanza un alto rendimiento de aislamiento: se estima que hay 50 veces más células madre en un gramo de tejido adiposo que en uno de médula ósea aspirada (Giai Via, Frizziero et al. 2012). Las ASCs se pueden obtener a partir de aspirados de liposucción o de grasa extirpada. Por otra parte, la plasticidad biológica de las ASCs es similar a la de médula ósea, siendo capaces de diferenciarse a osteoblastos, adipocitos, miocitos y condrocitos (Kolf, Cho et al. 2007).

Sin embargo, los diferentes tipos de MSCs también tienen diferencias de marcadores fenotípicos, y la funcionalidad biológica podría estar sujeta al ambiente fisiológico del nicho de origen, lo que podría distorsionar sus efectos en caso de obtenerse a partir de personas con obesidad o síndrome metabólico, patologías caracterizadas por una inflamación crónica sistémica (Pérez, de Lucas et al. 2017).

1.2 Potencial inmunomodulador

Durante los últimos años los efectos inmunoduladores, regenerativos y antiinflamatorios de las MSCs se han estudiado y comprobado extensivamente. En primer lugar, parece demostrado que el ambiente —ya sea inflamatorio o antiinflamatorio— tiene un efecto importante sobre el fenotipo y la función de las MSCs, por lo que estas células han sido propuestas como sensores de la inflamación, ya que adoptan distintos fenotipos de manera dinámica para modular y mantener la fisiología del tejido (Bernardo y Fibbe 2013). Es interesante destacar que la presencia de condiciones inflamatorias mejora su capacidad antiinflamatoria. Asimismo, su cultivo en presencia de distintos agentes proinflamatorios como interferón (IFN)-γ o interleucina (IL)-1β aumenta su potencial inmunosupresor (Contreras, Figueroa et al. 2016).

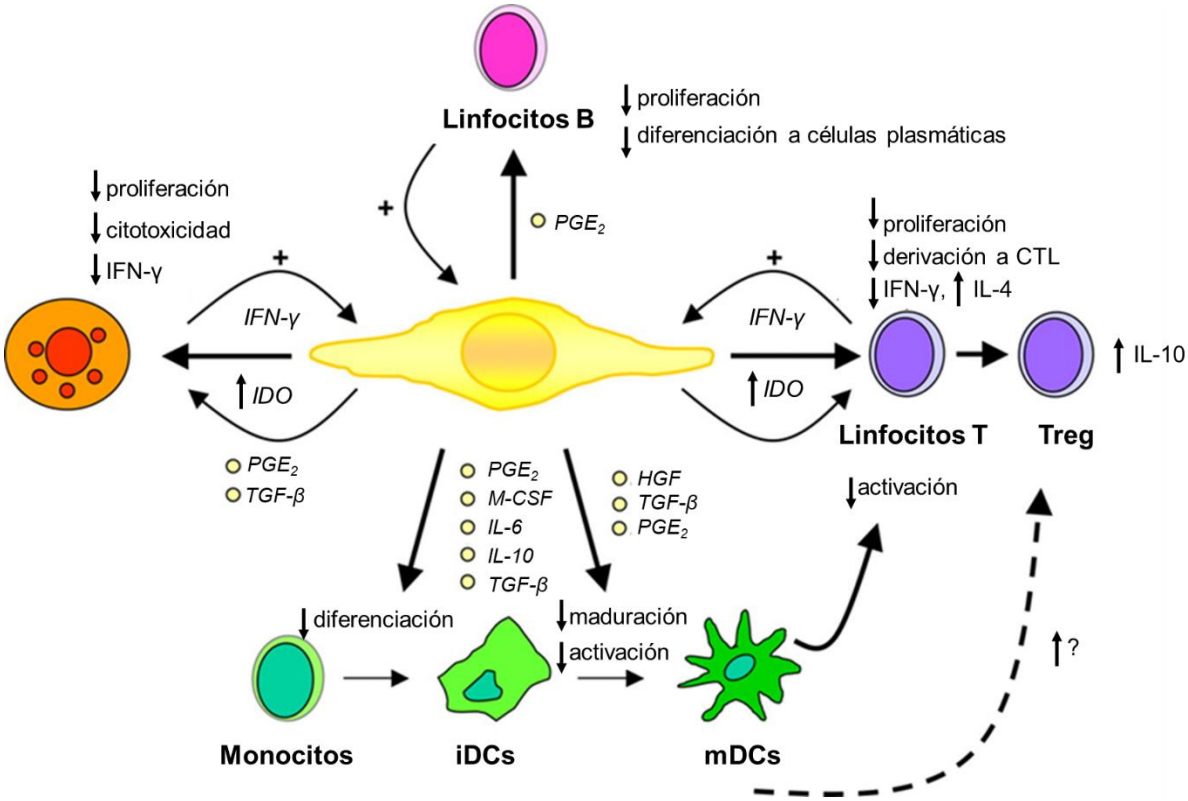

Figura 1. Efectos inmunomoduladores de las MSCs. PGE_2 indica prostaglandina E_2; IFN-γ, interferón-γ; IDO, indoleamina 2,3-dioxigenasa; TGF-β, factor de crecimiento transformante-β; M-CSF, factor estimulante de colonia de macrófagos; HGF, factor de crecimiento hepático; IL, interleucina; CTL, célula T citotóxica; Treg, célula T reguladora; iDC, célula dendrítica inmadura; y mDC, célula dendrítica madura. Modificado de Nauta y Fibbe 2007.

Los estudios de funcionalidad realizados hasta ahora parecen indicar que las MSCs son capaces de actuar tanto a nivel de la inmunidad innata como adquirida, promoviendo tanto la inhibición de respuestas inflamatorias como la activación y proliferación de fenotipos reguladores en otros tipos celulares que intervienen en la respuesta inmunitaria (Gebler, Zabel et al. 2012, Contreras, Figueroa et al. 2016). Así, la terapia con MSCs reduce la respuesta de linfocito cooperador 1/17, de célula B, de célula *natural killer* (NK) y de células dendríticas; al mismo tiempo, potencia a los macrófagos M2 y a los linfocitos T reguladores, y promueve la liberación de la citocina antiinflamatoria IL-10 (MacDonald, Augello et al. 2011). Estos efectos pueden estar mediados por el contacto celular directo de la MSC con su célula diana o bien a partir de efectores solubles (Contreras, Figueroa et al. 2016). En el primer caso, varios investigadores han mostrado el importante papel de distintas proteínas de adhesión como la molécula de adhesión intercelular (ICAM)-1 o la molécula de adhesión celular vascular (VCAM)-1 en los efectos inmunosupresores de las MSCs (Ren, Zhao et al. 2010). En cuanto a los mediadores solubles, las MSCs secretan una amplia variedad que incluye citocinas como IL-6 e IL-10, quimiocinas como la

quimiocina de tipo C-ligando (CCL)2, óxido nítrico (NO), purinas, proteínas morfogénicas de hueso, prostaglandina (PG)E_2, hemo oxigenasa-1, galectinas y factores de crecimiento como el factor de crecimiento transformante (TGF)-β1, el factor de crecimiento hepático (HGF), el factor de crecimiento vascular endotelial (VEGF) y el factor de crecimiento de fibroblastos (FGF) (Abbasi-Malati, Roushandeh et al. 2018). Además de estas moléculas solubles, las MSCs liberan una elevada cantidad de vesículas extracelulares (EVs), heterogéneas y de compleja composición bioquímica.

Todas estas características han llevado al estudio de nuevas terapias con MSCs en diversas patologías de las articulaciones con el objetivo, principalmente, de tratar y regenerar las lesiones condrales u óseas derivadas de un trauma o de la degradación inflamatoria crónica propia de varias patologías reumáticas. En lo que respecta a la reparación del cartílago, las MSCs se pueden inyectar en el espacio articular sin más, o bien implantarse en un biomaterial como andamio (*scaffold*) u otras construcciones de ingeniería tisular para imitar, hasta donde sea posible, la arquitectura fisiológica del espacio articular (Rai, Dilisio et al. 2017). En este proceso, las MSCs se dirigirían al tejido lesionado de forma selectiva y se diferenciarían en distintos tipos celulares, modulando el estado inflamatorio y sintetizando los componentes extracelulares necesarios para reparar la lesión (De Bari y Roelofs 2018).

Dado que sólo un pequeño porcentaje de las MSCs inyectadas en terapias locales permanecen en el sitio de la lesión, la eficacia regenerativa de estas células se ha atribuido a la secreción paracrina de distintos factores (Raposo y Stoorvogel 2013). Esta mezcla de componentes, denominada secretoma y estudiada *in vitro* como medio acondicionado (CM), sería la protagonista de la reparación del tejido al modular el microambiente local y promover la proliferación celular en él. Sin embargo, la composición de este secretoma es altamente compleja, heterogénea y variable en función del contexto de las MSCs y su tejido de procedencia (Murphy, Moncivais et al. 2013). Sus propiedades regenerativas se han estudiado y confirmado en varios modelos experimentales (Raik, Kumar et al. 2018). En condrocitos osteoartríticos, por ejemplo, el CM de las ASCs es capaz de inhibir la producción de mediadores degradativos (Platas, Guillén et al. 2013) y el desarrollo de senescencia celular (Platas, Guillén et al. 2016). El CM disminuye la expresión de distintos mediadores inflamatorios como IL-1β o metaloproteinasas de matriz (MMPs) en explantes sinoviales, así como la producción de NO en explantes de cartílago (van Buul, Villafuertes et al. 2012).

Estas evidencias indican que el contenido del CM derivado de MSCs podría tener aplicaciones terapéuticas interesantes en el tratamiento de enfermedades crónicas de las articulaciones. Por tanto, la atención se ha polarizado hacia la caracterización del contenido de este secretoma, tanto a nivel de su composición como de funcionalidad biológica.

VESÍCULAS EXTRACELULARES

2.1 Definición y nomenclatura

Las EVs comprenden un heterogéneo grupo de estructuras membranosas cerradas subcelulares que son liberadas por las células de forma evolutivamente conservada, tanto en organismos procariotas como en eucariotas superiores y plantas. Están presentes de manera ubicua en los fluidos biológicos y se han visto involucradas en multitud de procesos tanto fisiológicos como patológicos, a través de su capacidad de transferir información de forma paracrina o endocrina, modulando la función de la célula receptora. Así, el intercambio de EVs es considerado, hoy en día, un mecanismo adicional de comunicación intercelular de metabolitos, lípidos, proteínas y material genético entre tipos celulares de muy diverso fenotipo (van Niel, D'Angelo et al. 2018). Las EVs constituyen un particular empaquetado de biomoléculas que proporciona tanto protección a la degradación como la posibilidad de enviar diferentes mensajeros simultáneamente, incluso a lugares remotos al origen vesicular, dotando a la célula de una maquinaria robusta y flexible de comunicación con su entorno, acomodable al contexto fisiológico concreto (Yáñez-Mo, Siljander et al. 2015).

Las EVs fueron observadas por primera vez en 1946 como partículas procoagulantes derivadas de plaquetas en el plasma por Chargaff y West (Chargaff y West 1946), y denominadas *polvo de plaquetas* por Wolf (Wolf 1967). En 1983, diversos estudios de ultraestructura mostraron que estas partículas eran liberadas a través de cuerpos multivesiculares (MVBs) que se fusionaban con la membrana plasmática durante la diferenciación de los eritrocitos inmaduros (Harding, Heuser et al. 1984; Johnstone, Adam et al. 1987). Más de una década después, Raposo y sus colaboradores introdujeron el término *exosoma* para referirse a unas vesículas capaces de presentar antígenos e

inducir respuestas en células T (Raposo, Nijman et al. 1996). En 2006-2007 se descubrió que las EVs contienen RNA, incluyendo miRNA; desde entonces se han aislado EVs derivadas de la mayoría de tipos celulares y fluidos biológicos como la saliva, orina, el fluido nasal y bronquial, líquido amniótico, leche materna, plasma, suero y fluido seminal (Yáñez-Mo, Siljander et al. 2015).

La creciente información disponible indica que el contenido, tamaño y composición de membrana de las EVs es altamente heterogéneo y dinámico, no sólo depende de su origen celular sino también del estado y las condiciones ambientales. Actualmente se han consensuado tres grandes grupos de vesículas bajo el término "EV", acuñado por la Sociedad Internacional de Vesículas Extracelulares (ISEV, http://www.isev.org), definidos según su ruta de biogénesis: cuerpos apoptóticos, microvesículas (MVs), también llamadas micropartículas celulares o ectosomas, y exosomas (EXs). Los cuerpos apoptóticos son liberados en el proceso de invaginación de la membrana plasmática durante la apoptosis; el grupo de MVs comprende un rango heterogéneo de tamaño y naturaleza bioquímica de vesículas liberadas directamente por gemación de la membrana plasmática; los EXs, por último, son vesículas intraluminales contenidas en los MVBs, liberadas al medio extracelular tras la fusión de los MVBs con la membrana plasmática (Colombo, Raposo et al. 2014).

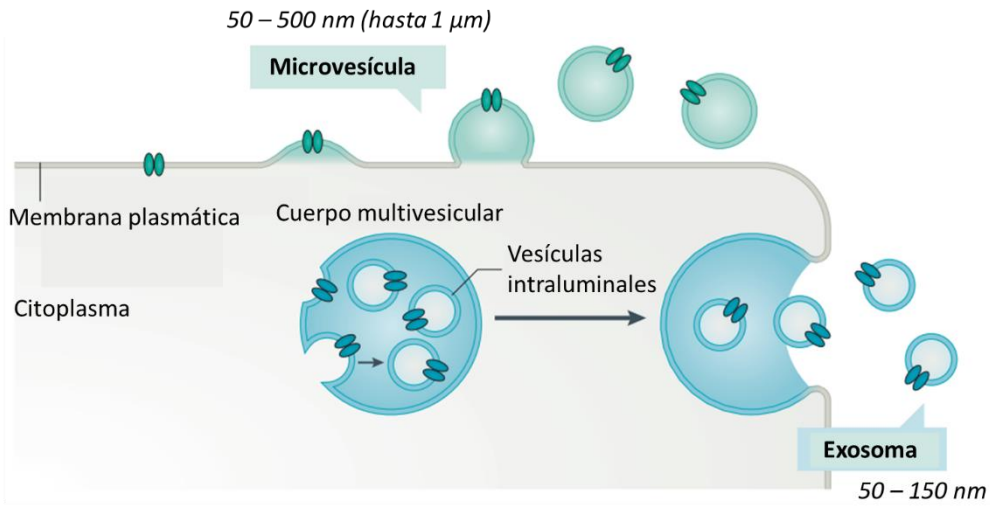

Figura 2. Características generales de microvesículas y exosomas. Las microvesículas se generan por gemación de la membrana plasmática y tienen un tamaño medio entre 50 y 500 nm; por su parte, los exosomas se generan a partir de los cuerpos multivesiculares de la ruta endocítica y tienen un tamaño entre 50 y 150 nm. Modificado de van Niel, D'Angelo et al. 2018.

Se han propuesto características y marcadores específicos para cada uno de los tres grupos, pero todavía no se ha aceptado ninguno que permita distinguirlos de manera excluyente. Del mismo modo, ninguna tecnología permite hasta el momento el aislamiento puro de ninguno de los grupos (Ramírez, Amorim et al. 2018). Por esta razón, existe un creciente interés en la búsqueda de una nomenclatura que permita, por un lado, unificar el criterio de los distintos grupos de investigación y, por otro, definir el objeto de estudio de forma inequívoca. A lo largo de los años, los investigadores han inventado docenas de nombres diferentes para las EVs, la mayoría de los cuales reflejan funciones específicas (vesículas calcificantes de matriz, que inician la biogénesis del hueso, o tolerosomas, que inducen tolerancia inmunológica), relevancia clínica (oncosomas, o vesículas inductoras de tumores (Quezada, Torres et al. 2017)) u origen (prostasomas liberados por el epitelio de la próstata (Gould y Raposo 2013)). Desgraciadamente, incluso los términos MV y EX, que podrían tener utilidad más amplia, también presentan dificultades, puesto que se han definido bajo dos criterios: en base a una definición biogenética (resumida en el párrafo anterior) y otra empírica, según el método de obtención en la práctica experimental; en especial, la velocidad de sedimentación de cada grupo (Théry, Amigorena et al. 2006, Théry, Ostrowski et al. 2009).

Por otra parte, esfuerzos más recientes por encontrar una nomenclatura universal han propuesto una división por tamaño junto con marcadores comúnmente encontrados en las preparaciones de EVs: (a) EVs grandes que precipitan a baja velocidad; (b) EVs de tamaño medio que precipitan a velocidad intermedia; y (c) EVs pequeñas (sEVs) que precipitan a alta velocidad. Entre estas sEVs se definirían cuatro subcategorías: (i) sEVs enriquecidas en CD63, CD9, CD81 así como otras tetraspaninas y marcadores endosomales; (ii) sEVs sin CD63 ni CD81 pero enriquecidas en CD9; (iii) sEVs sin CD63, CD9 ni CD81; y (iv) sEVs enriquecidas en factores derivados del suero o la matriz extracelular. De estas cuatro subcategorías, la primera correspondería con las EVs tradicionalmente denominadas EXs; la segunda vendría asociada a la membrana plasmática y una forma endocítica temprana; y las dos últimas no estarían asociadas a la ruta endosomal con formación de MVBs (Kowal, Arras et al. 2016).

La nomenclatura más comúnmente encontrada en la literatura suele combinar las definiciones biogenética y empírica: MVs como aquellas vesículas precipitadas a 12.600g tras una filtración a 0,8 μm, con enriquecimiento de aquellas partículas originadas por gemación de la membrana plasmática; y EXs como las EVs precipitadas tras una filtración a 0,2 μm seguida de una ultracentrifugación a 100.000g, enriquecidas en vesículas de origen endosomal. Los cuerpos apoptóticos, aunque están incluidos formalmente entre las EVs, no se suelen estudiar conjuntamente a las anteriores dadas sus diferencias morfológicas y funcionales (van Niel, D'Angelo et al. 2018).

2.2 Biogénesis y secreción de vesículas extracelulares

Los conocimientos acerca de los mecanismos generales de biogénesis de las distintas EVs son irregulares en función del campo de investigación y el tipo de vesícula. Esto no ocurre con la apoptosis o muerte celular programada ya que es un proceso exhaustivamente descrito desde hace décadas (Elmore 2007).

La biogénesis de EXs puede ocurrir de manera dependiente e independiente de los complejos endosomales de clasificación requeridos para el transporte (ESCRTs) (Fig. 3). Tanto en un caso como en el otro, el proceso comienza con la formación del MVB, proceso regulado a través de la ruta endocítica que comprende diversos compartimentos membranosos, altamente dinámicos, involucrados en la internalización de ligandos extracelulares o componentes celulares; su reciclado a la membrana plasmática y/o su degradación (Gould y Lippincott-Schwartz 2009, Klumperman y Raposo 2014). Durante el proceso de maduración de los endosomas tempranos a tardíos, estos acumulan vesículas de pequeño tamaño en su lumen, por lo que se suelezn denominar MVBs. Las vesículas que se forman por invaginación de las membranas endosomales tempranas secuestran proteínas, lípidos y moléculas citosólicas de manera específica. En la mayoría de las células, el destino principal de los MVBs es fusionarse con lisosomas, cargadas de hidrolasas ácidas que degradan su contenido. Sin embargo, los MVBs que contienen la tetraspanina CD63, proteínas de membrana asociadas a lisosomas (LAMPs) como LAMP1 y LAMP2, y otras moléculas generalmente presentes en los endosomas tardíos (como moléculas del complejo mayor de histocompatibilidad (MHC) de tipo II en células presentadoras de antígenos), se pueden fusionar también con la membrana plasmática, liberando su contenido al espacio extracelular (Raposo, Nijman et al. 1996, Jaiswal,

Andrews et al. 2002). En una misma célula coexisten distintas subpoblaciones de MVBs, algunas de ellas destinadas a la ruta de degradación y otras a la exocitosis (Vidal y Stahl 1993, Colombo, Raposo et al. 2014).

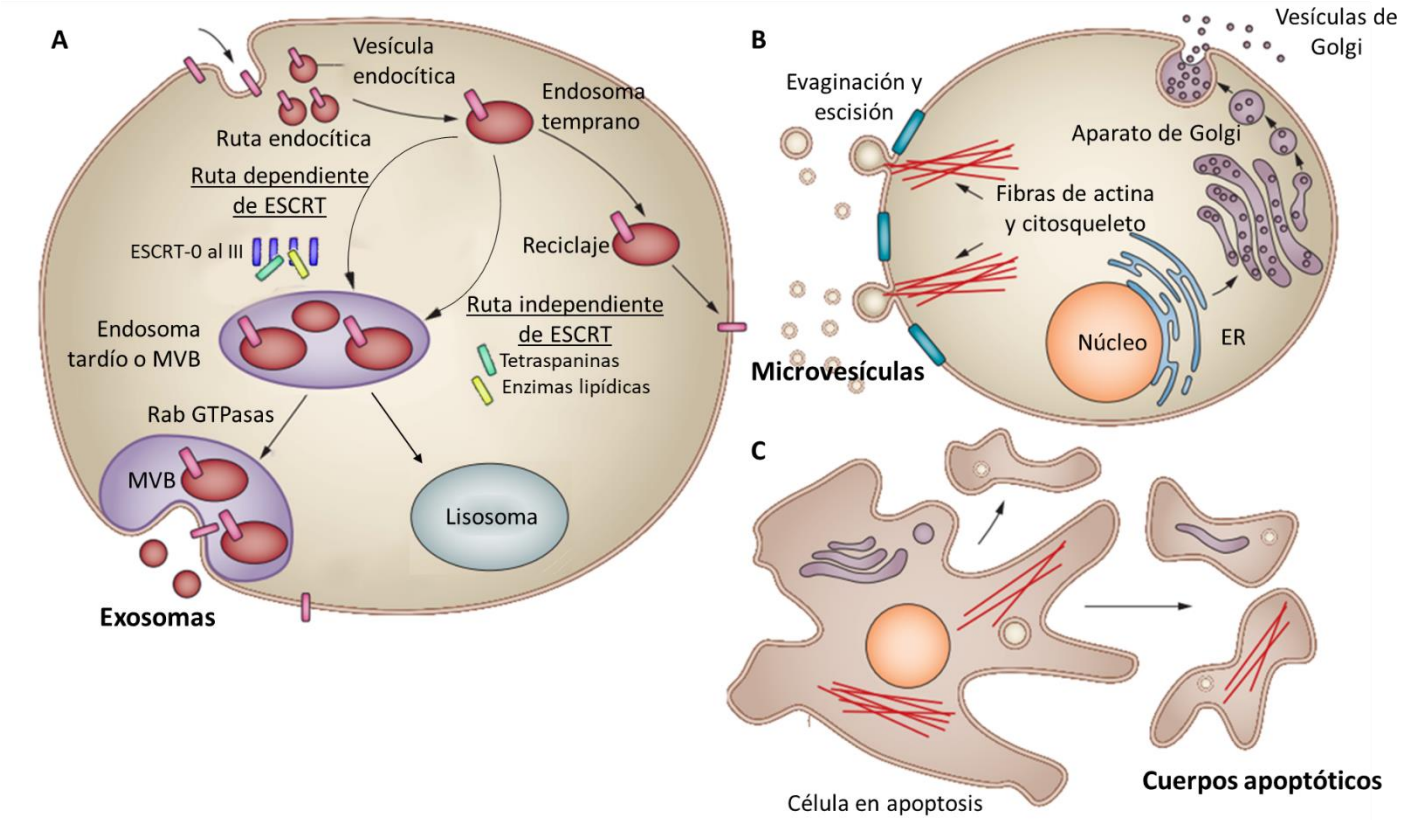

Figura 3. Biogénesis de las vesículas extracelulares. Las EVs se originan mediante distintos mecanismos. **(A)** Los exosomas se originan a partir de vesículas intraluminales formadas por endocitosis en respuesta a distintos estímulos; estas vesículas endocíticas maduran a endosomas tempranos, y estos a su vez a endosomas tardíos o MVBs mediante mecanismos que pueden depender o no de los complejos ESCRT. Tras interacciones entre las proteínas ESCRT y ubiquitina, los MVBs pueden derivar hacia la liberación de exosomas o hacia su degradación lisosomal. **(B)** Las microvesículas se forman por la gemación y fisión de microdominios lipídicos de la membrana, que están controladas por proteínas reguladoras y elementos del citoesqueleto que promueven la curvatura de la membrana en dominios enriquecidos en ceramida (barras azules). Al mismo tiempo, y tras su síntesis en el retículo endoplásmico (ER), determinadas proteínas específicas son transportadas al aparato de Golgi, donde son modificadas y empaquetadas en pequeñas vesículas de Golgi. **(C)** Las células en proceso de disgregación apoptótica producen grandes vesículas de membrana denominadas cuerpos apoptóticos. Modificado de Nawaz, Camussi et al. 2014.

El sistema ESCRT está compuesto de unas treinta proteínas ensambladas en cuatro complejos (ESCRT-0 al III) con distintas funciones, desde el reconocimiento de proteínas ubiquitinadas en la membrana endosomal, la deformación en yemas e introducción

selectiva de proteínas y otros compuestos, a las vesículas en formación hasta su escisión mediada por ESCRT-III (Hanson y Cashikar 2012). Muchas de las proteínas descritas en EVs forman parte o colaboran con estos complejos, como la proteína de interacción con muerte celular programada 6 (Alix), la clatrina, el gen de susceptibilidad a tumores 101 (Tsg101), la proteína 8 70kDa de estrés térmico (Hsc70), CD63 y otras tetraspaninas, así como diversas enzimas (Colombo, Raposo et al. 2014). Por su parte, la biogénesis independiente de ESCRT involucra a distintas proteínas en mecanismos variados y peor descritos. Por ejemplo, la tetraspanina CD63 o dos enzimas del metabolismo lipídico como la esfingomielinasa neural o la fosfolipasa D_2, pueden inducir procesos de gemación sin ESCRT (Stuffers, Sem Wegner et al. 2009, Colombo, Raposo et al. 2014). Ambos procesos confluyen en la fusión del MVB con la membrana plasmática y la liberación de los EXs, mediada por proteínas relacionadas con Ras (Rab) GTPasas (Nawaz, Camussi et al. 2014).

La biogénesis de MVs se ha estudiado con menor detalle y de manera más fragmentaria en distintos modelos celulares. En términos generales, sucede a partir de la evaginación y escisión de una porción de la membrana plasmática, habitualmente enriquecida en ceramida (Nawaz, Camussi et al. 2014), liberando la vesícula al espacio extracelular. La formación de estas yemas en la membrana está acompañada de cambios específicos locales en los componentes lipídicos y proteicos, modulando la curvatura de la membrana y su rigidez, así como el contenido de la futura vesícula (Tricarico, Clancy et al. 2017). El proceso comparte algunos componentes del sistema ESCRT, como Tsg101, que podría inducir cambios en la curvatura de la membrana, y ARF (*Ras-related GTPase ADP-ribosylation factor*) 6 (Nabhan, Hu et al. 2012) que dirige el reclutamiento selectivo de proteínas tales como la proteína de membrana asociada a vesícula (VAMP) 3, la integrina β1 o el MHC-I (Muralidharan-Chari, Clancy et al. 2009). De hecho, parece cada vez más claro que, al igual que los EXs, las MVs no son simplemente muestras aleatorias del contenido citoplasmático, sino que el reclutamiento de proteínas y ácidos nucleicos es activo y selectivo, al menos a cierto nivel (D'Souza-Schorey y Clancy 2012, Roberts y Kurre 2013). Por último, la fisión y liberación de la vesícula es dependiente de la interacción entre actina y miosina y la consiguiente contracción dependiente de ATP (D'Souza-Schorey y Clancy 2012). Se sospecha que ARF6 también tiene un papel muy importante en este proceso, ya que activa a la quinasa regulada por señal extracelular (ERK) a través de la fosfolipasa D, lo que lleva a la activación de una ruta de quinasas

que confluyen en la fosforilación de la cadena ligera de miosina y la activación de esta maquinaria contráctil (Muralidharan-Chari, Clancy et al. 2009, Tricarico, Clancy et al. 2017). Otro pequeño subgrupo de MVs se producen a nivel del aparato de Golgi a partir de proteínas específicas sintetizadas en el retículo endoplásmico, donde son marcadas y dirigidas hacia el aparato de Golgi, empaquetadas, modificadas y liberadas como vesículas (Nawaz, Camussi et al. 2014).

2.3 Composición bioquímica y funcionalidad

2.3.1 Proteínas

El contenido proteico de las EVs y otras vesículas ha sido estudiado exhaustivamente desde su descripción inicial. En primer lugar se emplearon técnicas basadas en detección con anticuerpos para proteínas específicas (*western blotting* y ensayo de inmunoabsorción ligado a enzima o ELISA), pero el desarrollo de las técnicas de análisis proteómico en la década de los 90 ha permitido la identificación a gran escala de proteínas en las preparaciones de EVs (Colombo, Raposo et al. 2014). Ya que los ensayos bioquímicos y la citometría de flujo sólo permiten estudiar proteínas conocidas, el énfasis actual se ha desplazado hacia la espectrometría de masas, que se realiza habitualmente en tándem con cromatografías líquidas, con la aplicación ocasional de electroesprays de ionización (Rosa-Fernandes, Rocha et al. 2017). Los estudios proteómicos de EVs obtenidas de distintas fuentes han resultado en la elaboración de extensos catálogos de contenido y concentración proteica en numerosas poblaciones de EVs, lo que ha permitido el desarrollo de bases de datos públicas que compendian la información disponible sobre EVs, como ExoCarta o su sucesora Vesiclepedia (http://microvesicles.org) (Yáñez-Mo, Siljander et al. 2015). Se han descrito proteínas que están presentes de manera relativamente universal en distintos tipos de EVs, así como modificaciones post-traduccionales que reflejan su localización vesicular, el origen celular y los mecanismos de secreción (Escrevente, Keller et al. 2011, Ostergaard, Nielsen et al. 2012). En general, las EVs contienen en abundancia proteínas de membrana plasmática, citosqueléticas, citosólicas, de tráfico vesicular y de respuesta a estrés, siendo menos frecuentes las proteínas de orgánulos intracelulares.

No obstante, los perfiles proteicos que se han obtenido hasta ahora son altamente dependientes de la metodología de aislamiento: los diferentes métodos obtienen EVs y

subpoblaciones de EVs con distinto rendimiento y homogeneidad variable, lo que dificulta la extrapolación de resultados de diferentes estudios proteómicos. Asimismo, es difícil distinguir entre las proteínas propiamente vesiculares —contenidas en el lumen de las EVs o embebidas en su membrana— de las denominadas asociadas a EVs (*EV-associated proteins*) que son co-aisladas y frecuentemente analizadas en numerosos estudios proteómicos (Rosa-Fernandes, Rocha et al. 2017). Además, una misma célula puede liberar distintas EVs en función del contexto fisiológico en el que se encuentra. En este sentido, todavía no se ha descrito ningún marcador que pueda identificar EVs únicamente y de forma universal. Por lo tanto, aún son necesarios nuevos trabajos que permitan ampliar y clarificar la información disponible (Yáñez-Mo, Siljander et al. 2015).

Como marcadores se suelen emplear proteínas tales como tetraspaninas (CD9, CD63, CD81 y CD82), la proteína 14-3-3, moléculas del MHC, proteínas citosólicas tales como Alix y otras específicas de estrés: estrés término (Hsc70) y estrés genómico (Tsg101) (Witwer, Buzás et al. 2013). Anteriormente se consideraba a las tetraspaninas CD9, CD63 y CD81 como marcadores específicos de EXs. Sin embargo, estas proteínas también se encuentran en cuerpos apoptóticos y MVs. En conjunto, CD9 y Alix son las proteínas más frecuentemente identificadas (Rosa-Fernandes, Rocha et al. 2017).

Las funciones efectoras de las proteínas contenidas en las EVs dependen, lógicamente, del tipo de vesícula y su célula de origen. Una vez liberadas al espacio extracelular, las EVs pueden alcanzar sus células diana y traspasar su contenido, provocándoles respuestas funcionales y cambios fenotípicos. La especificidad de diana suele estar determinada por la abundancia de proteínas concretas que interactúan con receptores en la membrana de las células diana. Se sabe que las tetraspaninas, integrinas, lípidos, lectinas y proteoglicanos pueden ejercer este papel, si bien los detalles moleculares de estas interacciones se desconocen en su mayor parte. Por otro lado, es importante recordar que la diana puede ser la propia célula productora, en forma de mecanismo autocrino (van Niel, D'Angelo et al. 2018). Tras la interacción con la membrana plasmática y su internalización, las EVs siguen la ruta endocítica y alcanzan los MVBs, donde suelen ser derivadas a la degradación lisosómica. En algunos casos, las vesículas escaparán a la digestión por fusión con la membrana del MVB, liberando su contenido al citoplasma (van Niel, D'Angelo et al. 2018).

Las proteínas de las EVs producidas por MSCs se han estudiado en algunos trabajos con resultados difíciles de integrar, debido a las distintas definiciones manejadas por los

investigadores. Muchas de las proteínas identificadas tienen capacidad inmunomoduladora o están relacionadas con mecanismos de producción de energía como la glicólisis, lo que ha reforzado su interés en distintas áreas biomédicas (Lai, Yeo et al. 2015).

Tabla 1. Proteínas exosomales citadas un mayor número de veces en la literatura científica.

Nombre reducido	Nombre completo	Nº de veces citado
CD9	Clúster de Diferenciación 9	98
Alix	Proteína de interacción con muerte celular programada 6	96
Hsc70	Proteína 8 70kDa de estrés térmico	96
GAPDH	Gliceraldehído-3-fosfato deshidrogenasa	95
β-Act	β-actina	93
Anx-A2	Anexina A2	83
CD63	Clúster de Diferenciación 63	82
Synt-1	Sintenina 1	78
Eno-1	Enolasa 1	78
Hsp90-α	Proteína de estrés térmico 90-α	77
Tsg101	Gen de susceptibilidad a tumores 101	75

2.3.2 Ácidos nucleicos

La presencia de RNA funcional en el interior de las EVs se describió por primera vez en 2006 en EVs derivadas de células madre murinas (Ratajczak, Miekus et al. 2006) y su transferencia a células receptoras en 2007 (Valadi, Ekstrom et al. 2007). Mientras que el RNA mensajero (mRNA) celular varía en tamaño entre 400 y 12.000 nucleótidos, el RNA detectado en EVs tiene un tamaño predominante de 700 nucleótidos. Las EVs contienen mRNA intacto, fragmentos de mRNA, RNA no-codificante largo, miRNA, *piwi-interacting RNA*, RNA ribosómico y fragmentos de tRNA, *vault*-RNA e Y-RNA (Yáñez-Mo, Siljander et al. 2015). Por otra parte, existe una creciente evidencia que indica que el RNA no entra pasivamente en las EVs, sino que es enriquecido en determinadas especies de manera activa y específica. En el caso de los mRNAs, se ha observado la existencia de una secuencia consenso en el 3' UTR que podría actuar como marca para su introducción en las EVs (Bolukbasi, Mizrak et al. 2012).

Ya se ha demostrado que las EVs transfieren mRNA funcional a sus células diana y que éste es capaz de expresarse (Deregibus, Cantaluppi et al. 2007). El contenido de mRNA en las EVs está modulado por el estado fisiológico de la célula y las condiciones de estrés, y podría tener un papel en el mantenimiento de la homeostasis tisular y la sincronización del estado funcional de las células. Sin embargo, sigue siendo difícil asociar una función a RNAs concretos dentro de la EV dada la compleja mezcla que contiene. Al mismo tiempo, no está claro qué parte del transcriptoma de las EVs está intacto y qué parte se encuentra fragmentado (Yáñez-Mo, Siljander et al. 2015).

Por su parte, los miRNAs son secuencias reguladoras de unos 21 nucleótidos que se transcriben como horquillas precursoras escindidas a pre-miRNAs por Dicer, los cuales se unen a las proteínas argonauta y son cargados al complejo silenciador inducido por miRNA, donde suelen ejercer su función interactuando con mRNAs específicos y, habitualmente, induciendo su degradación. Los miRNAs son secretados tanto dentro de EVs como asociados a complejos proteicos solubles (Yáñez-Mo, Siljander et al. 2015). La acumulación de miRNAs en las EVs les permite circular en la sangre sin degradarse. Asimismo, el traspaso selectivo de algunos miRNAs contenidos en las EVs podría ser una manera rápida de regular la expresión génica en diversos procesos. En este sentido, se ha observado que los miRNAs contenidos en EXs de MSCs de médula ósea fueron capaces de inducir la diferenciación osteogénica de osteoblastos (Xu, Yang et al. 2014).

Por último, en contraste con el RNA, la presencia de DNA en las EVs se ha explorado menos hasta ahora. No obstante, se ha encontrado DNA de cadena simple, DNA mitocondrial, DNA de doble cadena y amplificaciones oncogénicas en las EVs (Yáñez-Mo, Siljander et al. 2015). Se ha propuesto la posibilidad de que la migración del DNA mitocondrial podría tener lugar vía EVs y, por tanto, que las EVs podrían representar una ruta alternativa de entrada del mtDNA en otras células, favoreciendo la difusión de diversas patologías (Guescini, Genedani et al. 2010).

El estudio del contenido en ácidos nucleicos implica desde tecnologías sencillas como la reacción en cadena de la polimerasa (PCR) en sus distintas modalidades, hasta el empleo de micromatrices de expresión o estrategias de secuenciación masiva (Yáñez-Mo, Siljander et al. 2015).

2.3.3 Lípidos

Comparativamente, existen muchos menos estudios que hayan abordado la composición lipídica de las EVs; la mayoría de ellos a través de ensayos bioquímicos y cromatografía de alto rendimiento, aunque cada vez se utiliza la espectrometría de masas con más frecuencia. Si bien ya se han encontrado diferencias entre las EVs de distintas fuentes, en general parecen estar enriquecidas en esfingomielina, colesterol, fosfatidilserina, ceramida y glicoesfingolípidos (Colombo, Raposo et al. 2014). Aparte de su indudable papel estructural, algunos trabajos han propuesto una función activa de algunos lípidos encontrados en las EVs. Las EVs transfieren lípidos bioactivos tales como eicosanoides, ácidos grasos y colesterol. Por ejemplo, las prostaglandinas unidas a la vesícula podrían activar numerosos mecanismos de señalización intracelular en las células diana (Yáñez-Mo, Siljander et al. 2015).

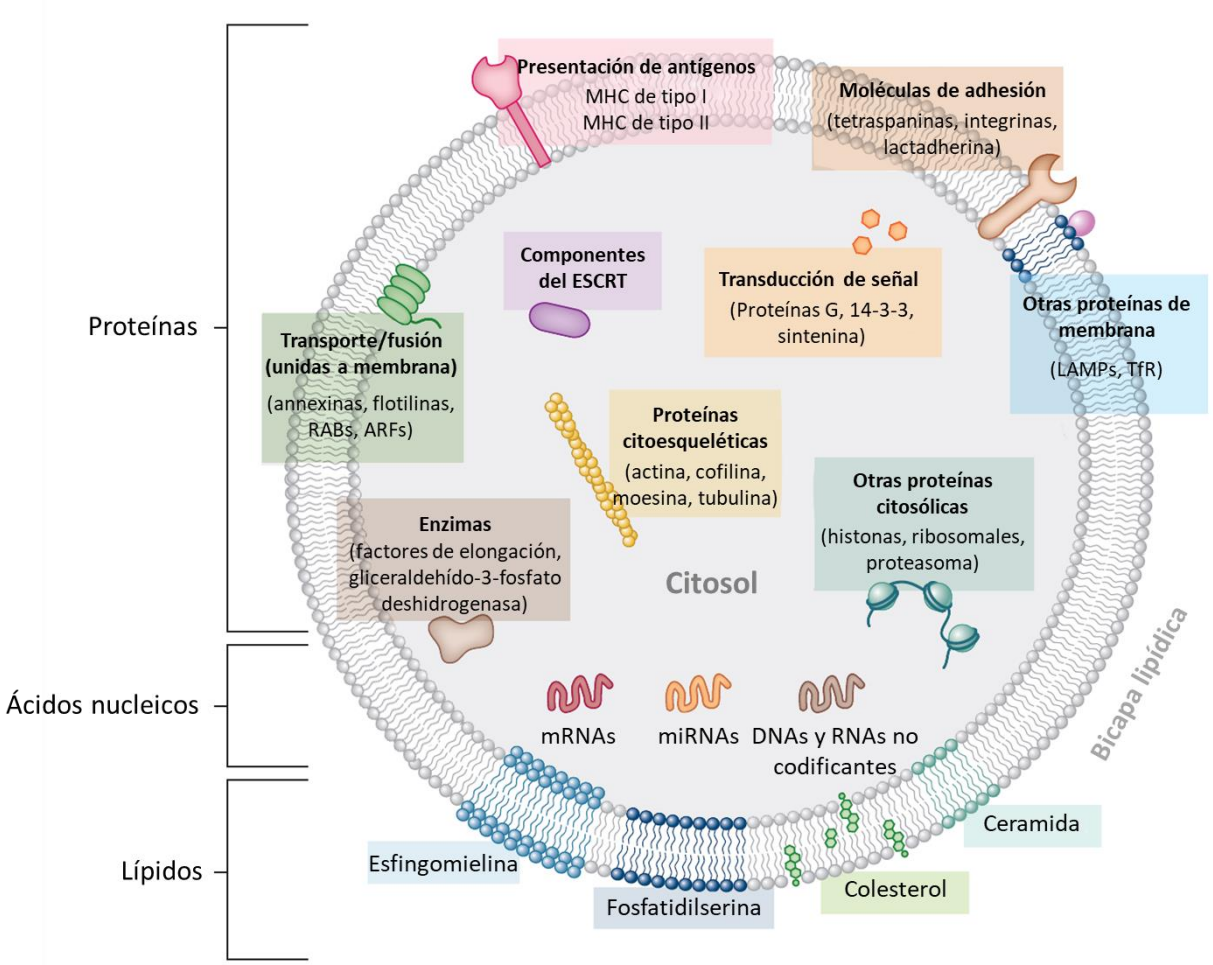

Figura 4. Composición general de las vesículas extracelulares. Representación esquemática de la composición, estructurada en familias de proteínas, lípidos y ácidos nucleicos, y la orientación de la membrana de las EVs. Ejemplos de tetraspaninas comúnmente encontradas en EVs son CD63, CD81 y CD9. La composición reflejada en la imagen es genérica por lo que

algunos componentes podrían estar sólo en algunos subtipos de EVs. ARF, *Ras-related GTPase ADP-ribosylation factor*; ESCRT, complejo endosomal de clasificación requerido para el transporte; MHC, complejo mayor de histocompatibilidad; LAMP, proteína de membrana asociada a lisosoma; Rab, proteína relacionada con Ras en cerebro; TfR, receptor de transferrina. Modificado de Colombo, Raposo et al. 2014.

2.4 Aislamiento y caracterización de vesículas extracelulares

Las EVs se obtienen a partir de una gran variedad de orígenes biológicos. La fase preanalítica es una fuente importante de variabilidad y puede contribuir a la aparición de artefactos, en especial si se parte de fluidos con presencia de restos celulares como plasma o suero, donde es imperativa la eliminación de plaquetas (Coumans, Brisson et al. 2017). También es importante la depleción de EVs presentes en los medios de cultivo (Shelke, Lässer et al. 2014) y la eliminación de las proteínas más abundantes para los estudios de caracterización proteica (Lacroix, Judicone et al. 2013).

2.4.1 Métodos de aislamiento

Existen distintas técnicas de aislamiento de EVs. Ninguna de ellas combina un elevado rendimiento con la ausencia de impurezas y la posibilidad de discriminar subpoblaciones vesiculares, por lo que es importante conocer las características de cada una para seleccionar la más adecuada según las exigencias experimentales concretas. Los métodos de aislamiento más utilizados son la centrifugación diferencial, la centrifugación en gradiente de densidad, la cromatografía de exclusión molecular, la precipitación mediante polímeros comerciales y la inmunocaptura magnética (Coumans, Brisson et al. 2017).

Entre todas ellas, la centrifugación diferencial es uno de los métodos más comunes y ampliamente usados para aislar EVs provenientes de CM y otros fluidos biológicos. A lo largo del tiempo se han propuesto y utilizado distintos protocolos, pero los pasos principales siguen incluyendo tres centrifugaciones seriadas a velocidades crecientes con filtraciones intermedias para eliminar la mayor proporción posible de contaminantes. Habitualmente comienza con una centrifugación a baja velocidad que precipita los restos celulares y los cuerpos apoptóticos, seguida de una centrifugación a velocidad media que precipita las MVs o vesículas de tamaño mediano y, por último, una ultracentrifugación a altas velocidades para el aislamiento de los EXs o vesículas de pequeño tamaño (Fig. 5) (Carpintero-Fernández, Fafián-Labora et al. 2017). El precipitado de EVs se resuspende entonces en un medio fisiológico, habitualmente tampón fosfato salino (PBS). Para aumentar la pureza de la muestra se puede realizar un lavado en grandes volúmenes

de PBS con la centrifugación apropiada. Sin embargo, aumentar el número de centrifugaciones puede comprometer la estabilidad de las vesículas, por lo que se debe buscar un equilibrio entre ambos factores (Momen-Heravi, Balaj et al. 2013).

La centrifugación diferencial se ha utilizado con éxito durante décadas en diversos procesos de aislamiento. Además, es capaz de discriminar por tamaño distintas subpoblaciones de EVs. Sus principales inconvenientes son la necesidad de ultracentrífuga, el tiempo de ejecución (en torno a 5-6 horas), la abundante co-precipitación de contaminantes proteicos y el rendimiento relativamente bajo de aislamiento, de entre un 5 y un 25% (Momen-Heravi, Balaj et al. 2013). Pese a estos problemas, sin embargo, continúa siendo una técnica de gran utilidad cuando se manejan grandes volúmenes de partida como en el caso del suero o el CM, y cuando las EVs deben conservar su funcionalidad fisiológica.

En comparación, la centrifugación en gradiente de densidad compromete la funcionalidad de las EVs (Paolini, Zendrini et al. 2016); la cromatografía de exclusión molecular no está adaptada todavía a volúmenes elevados ni permite discriminar subpoblaciones de EVs por tamaño (Taylor y Shah 2015); la precipitación con polímeros produce preparaciones muy impuras, poco adecuadas para estudios de funcionalidad (Taylor, Zacharias et al. 2011) y la inmunocaptura magnética tampoco proporciona rendimientos suficientes si la muestra de partida está diluida (Pedersen, Kierulf et al. 2017).

2.4.2 Caracterización física: tamaño y concentración

Las técnicas más frecuentemente utilizadas para caracterizar EVs son el *western blotting* y el ELISA. Sin embargo, ninguno de estos métodos proporciona información sobre la estructura, concentración o heterogeneidad de las EVs (Ostrowski, Carmo et al. 2010). Por esta razón, durante los últimos años se ha hecho gran hincapié en el desarrollo de nuevas tecnologías que permitan ampliar el conocimiento sobre las EVs, tanto a nivel de su tamaño, heterogeneidad y concentración, como de su contenido, ya sea proteico, genómico, lipídico o metabólico (Carpintero-Fernández, Fafián-Labora et al. 2017).

Existen diversos métodos que permiten determinar el tamaño y concentración de EVs en una muestra, dentro de los cuales los más comunes son la citometría de flujo (FC, *Flow Cytometry*), el análisis de rastreo de nanopartículas (NTA, *Nanoparticle Tracking Analysis*) y la detección de pulso resistivo ajustable (TRPS, *Tunable Resistive Pulse Sensing*).

El RPS, también conocido como efecto Coulter, describe el cambio en la resistencia entre dos electrodos cuando una partícula no conductora atraviesa una apertura que separa los electrodos, que se encuentran sumergido en una solución de electrolitos para permitir el flujo de corriente entre ellos. Conforme las partículas atraviesan la abertura, desplazan un cierto volumen del electrolito, cambiando la resistencia del circuito y causando una caída en la corriente, lo que produce un "pulso resistivo". El tamaño de este pulso es linealmente proporcional al volumen de la partícula que atraviesa la apertura, lo que permite calcular el tamaño medio de las partículas en la suspensión (Buzás, Gardiner et al. 2017).

Recientemente se ha desarrollado una nueva tecnología, el TRPS, que permite la detección del tamaño y la concentración de partículas muy pequeñas con una elevada precisión empleando un poro submicrométrico encajado en una membrana elástica (Fig. 5) (Sowerby, Broom et al. 2007). Dada la flexibilidad de la membrana, el poro puede ser ajustado dinámicamente, cambiando su tamaño y propiedades conductivas para optimizar el estudio de muestras variadas, e incluso discriminar entre distintas poblaciones en un mismo experimento (Roberts, Kozak et al. 2010). Mediante el control preciso del flujo de partículas a través del sistema, variando el flujo y la carga en el equipo y empleando partículas de calibración, se puede determinar el tamaño de partícula, la concentración y las características de carga, como el potencial Zeta, que describe la intensidad de campo eléctrico de la membrana. Los equipos comerciales de TRPS suelen tener un umbral de detección en torno a los 40 nm y un límite superior determinado por el tamaño del poro. Si bien el TRPS es una tecnología relativamente nueva, diversos autores han concluido que su capacidad de cuantificación es, al menos, similar a la de otras técnicas como el NTA o la FC, y se ha utilizado recientemente como método estándar para evaluar el rendimiento de distintos procedimientos de aislamiento de EXs (Lane, Korbie et al. 2015, Buzás, Gardiner et al. 2017). Entre sus ventajas destaca su relativa simplicidad y su precisión, así como su capacidad de discriminar distintas poblaciones y su flexibilidad operativa. Sus limitaciones más importantes tienen que ver con la frecuente obstrucción del poro cuando se trabaja en condiciones de alto rendimiento o con EVs de tamaños muy diversos. Esta obstrucción requiere detener el experimento para limpiar el sistema, y puede llegar a alterar la naturaleza del poro de maneras impredecibles (Coumans, van der Pol et al. 2014). Por otra parte, aunque caracteriza los parámetros físicos de las EVs extremadamente bien, no proporciona información acerca del contenido químico y no permite ninguna técnica de marcaje específico (Buzás, Gardiner et al. 2017).

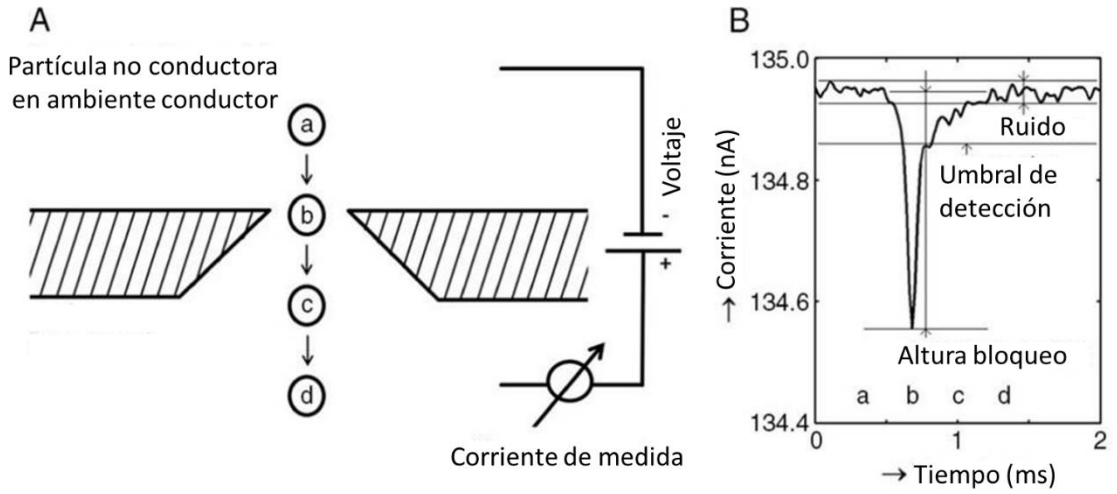

Figura 5. Principio operativo del TRPS. Cuando una partícula no conductora atraviesa un medio conductor a través de un poro, se produce un breve incremento en la resistencia eléctrica del poro. La intensidad de bloqueo y el flujo de partículas permiten inferir el tamaño y la concentración, respectivamente, de las partículas analizadas. **(A)** Representación esquemática del nanoporo con una vesícula haciendo un recorrido a-d. **(B)** Bloqueo de la corriente debido al paso de una partícula. Las letras a-d corresponden a las posiciones de las vesícula en A. Modificado de Coumans, van der Pol et al. 2014.

De los métodos mencionados hasta ahora, tan sólo la FC permite diferenciar entre EVs y partículas no vesiculares; sin embargo, su resolución a diámetros bajos es escasa, por lo que son necesarias técnicas adicionales para identificar las propiedades estructurales y visualizar vesículas individuales. Las guías de investigación en EVs suelen recomendar la combinación de ambos tipos de técnicas para alcanzar la máxima comprensión posible de las EVs en estudio (Coumans, Brisson et al. 2017).

Todos los tipos de EVs se pueden detectar y caracterizar al nivel de partícula única mediante microscopía electrónica (EM) en la que se utilizan haces de electrones en lugar de fotones visibles para configurar imágenes de objetos nanoscópicos (Szatanek, Baj-Krzyworzeka et al. 2017). La microscopía electrónica de transmisión y la de barrido son las dos herramientas estándar para caracterizar la morfología de las EVs: en el primer caso, se obtienen imágenes de la morfología y ultraestructura de la vesícula; en el segundo, de la topografía superficial. Además, la EM se puede combinar con un inmuno-marcaje específico con anticuerpos conjugados a nanopartículas de oro de distinto tamaño, lo que permite la detección de una o más proteínas de EVs (Carpintero-Fernández, Fafián-Labora et al. 2017). Sin embargo, la EM requiere una alta

manipulación de la muestra que incluye fijación, deshidratación, revestimiento y teñido, lo que afecta a la estructura general de la EV. De hecho, hoy en día se sabe que la morfología ovoide tradicional asociada a las EVs es un artefacto debido al procesamiento, ya que se aplasta la vesícula y genera esa forma. Para mantener la morfología nativa se ha desarrollado una versión criogénica de la EM (Coumans, Brisson et al. 2017).

En la actualidad se están adaptando otras técnicas para caracterizar el tamaño, morfología y concentración de las EVs con algunas ventajas o prestaciones frente a las tradicionales. Entre ellas destaca la microscopía de fuerza atómica que permite explorar la topografía superficial de la membrana con una gran resolución (Whitehead, Wu et al. 2015) y la espectroscopía de Raman, que puede potencialmente caracterizar tamaño, morfología y contenido en un mismo experimento (Buzás, Gardiner et al. 2017).

2.5 Aplicaciones biomédicas de las vesículas extracelulares

Debido a la presencia ubicua de EVs en prácticamente todos los entornos y fluidos biológicos, estas vesículas han recibido una notable atención por sus prometedoras aplicaciones biomédicas como herramientas de diagnóstico, transportadores de moléculas bioactivas, dianas o agentes terapéuticos.

Las ventajas de las EVs para el diagnóstico de distintas patologías son indudables. Su biodisponibilidad es alta: se han encontrado elevadas concentraciones de EVs en saliva, orina y sangre, tres fluidos cuya obtención resulta sencilla. Las EVs que contienen son fáciles de aislar con los métodos existentes y, lo que es más, responden de manera muy sensible a los cambios fisiológicos del organismo, lo que ha permitido encontrar marcadores precoces en numerosas patologías tales como diversos cánceres, enfermedades hepáticas, cardiovasculares y de origen inflamatorio (Whiteside 2017). Dado que muchos de los equipos necesarios para el aislamiento están disponibles en la mayoría de los hospitales y que muchos de los problemas relacionados con la obtención de EVs en investigación, como la pureza o la estabilidad bioquímica, no son tan relevantes para el diagnóstico, es posible que se desarrollen aplicaciones diagnósticas basadas en EVs en un futuro próximo (Armstrong y Wildman 2018).

Las EVs no sólo tienen un papel fisiológico, sino que también pueden ser agentes patológicos. Se sabe que las EVs liberadas por células tumorales son capaces de inducir

la tumorogénesis en otras células diana, así como el ambiente tolerogénico que impide el funcionamiento correcto del sistema inmune (Sundararajan, Sarkar et al. 2018). En enfermedades cardiovasculares como la aterosclerosis, las EVs transportan enzimas proteolíticas que agravan la enfermedad. En estos casos, utilizar a las EVs como diana terapéutica parece una opción prometedora, donde el objetivo sería el bloqueo de la transferencia de su contenido a través de distintos mecanismos (van der Vorst, de Jong et al. 2018).

Por otra parte, las EVs producidas por distintos tipos celulares, en especial células madre hepáticas y MSCs, se han propuesto como agentes terapéuticos potenciales en distintas patologías. Una terapéutica basada en EVs podría utilizar las vesículas directamente, o bien emplearlas como transportadoras de moléculas bioespecíficas introducidas *ad hoc* en el lumen. Además, el uso de EVs presenta ciertas ventajas frente a las terapias celulares: se pueden administrar de forma similar a los fármacos convencionales, pueden cruzar barreras biológicas que otros sistemas terapéuticos no pueden, minimizan el riesgo oncológico derivado de la multipotencia de muchas células madre y también el contagio de virus empleados en ingeniería genética. Asimismo, dado que las EVs son menos inmunogénicas, la posibilidad de reacciones inmunitarias adversas es más limitada (Raik, Kumar et al. 2018).

Sin embargo, en la actualidad el uso de EVs como agentes terapéuticos presenta muchos desafíos. Entre ellos, destacan las metodologías utilizadas en el aislamiento, difícilmente escalables en muchos casos o cuyo resultado no es lo suficientemente definido en otros. Además, la inexistencia de una nomenclatura universal confunde la información preclínica disponible. Por otra parte, apenas se dispone de información sobre la concentración local o la vida media de las vesículas en distintos fluidos biológicos (Raik, Kumar et al. 2018).

2.5.1 EVs y artrosis

Una de las aplicaciones más prometedoras de las EVs reside en el área de las enfermedades inflamatorias crónicas, donde el efecto inmunomodulador y regenerativo asociado a las vesículas producidas por MSCs podría resultar de gran interés. Así, las enfermedades reumáticas representan una de las áreas donde las EVs podrían aportar más posibilidades.

La OA o artrosis es la patología de las articulaciones más común en Occidente, relacionada con procesos de envejecimiento y considerada la principal causa de dolor e incapacidad física en las personas de edad avanzada. Se caracteriza por una destrucción del cartílago articular acompañada de hipertrofia ósea con formación de osteofitos, esclerosis del hueso subcondral, inflamación crónica de la membrana sinovial y disminución del espacio articular. Estos cambios estructurales se manifiestan con dolor, rigidez, deformación y limitación funcional de la articulación (Fig. 6) (Martel-Pelletier, Barr et al. 2016). A pesar de que la etiología de la enfermedad no está completamente definida, los factores de riesgo más comúnmente asociados a ella son la edad, el sexo femenino, la obesidad, la presencia de lesiones previas en la articulación, cierta predisposición genética y distintos factores mecánicos (Loeser, Goldring et al. 2012). La gran problemática de la OA radica en que, a día de hoy, las terapias disponibles están dirigidas a aliviar la sintomatología sin impedir la degradación progresiva del cartílago y el hueso subcondral, lo que hace imperativa la búsqueda de nuevas estrategias y dianas terapéuticas (Martel-Pelletier, Wildi et al. 2012).

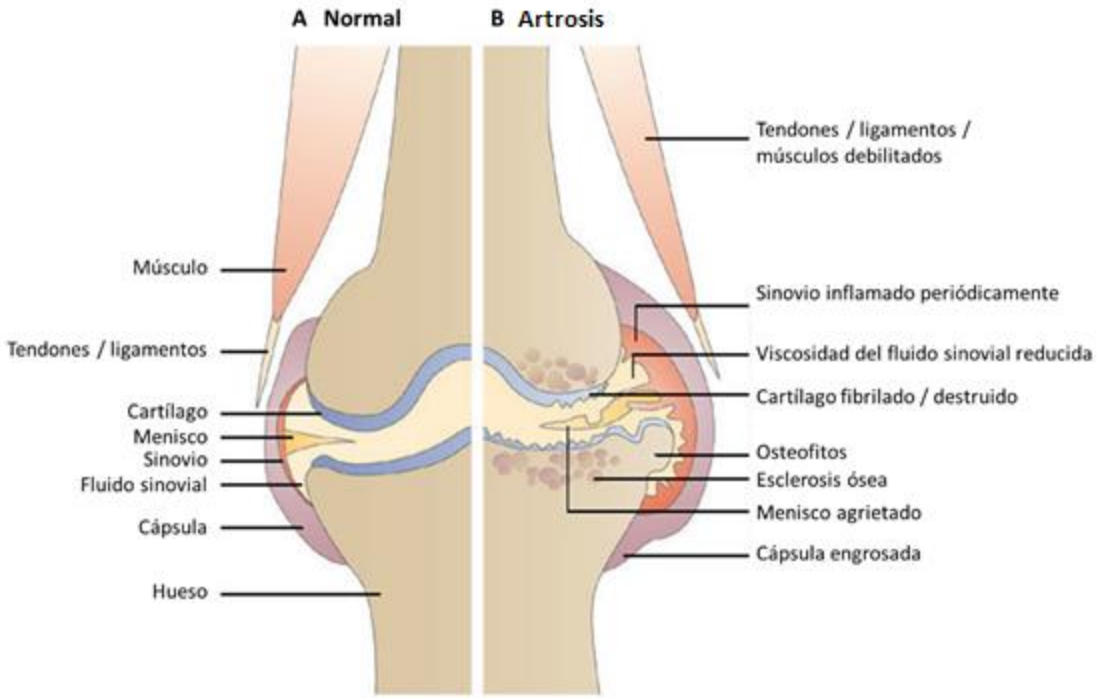

Figura 6. Estructuras articulares afectadas en la OA. (A) Articulación sana. **(B)** Artrosis: lesión temprana degenerada con cartílago fibrilado y remodelación de hueso, lo que lleva a la formación de osteofitos, inflamación sinovial y esclerosis subcondral. Modificado de Wieland, Michaelis et al. 2005.

El cartílago articular es una forma de cartílago hialino que actúa como recubrimiento de las epífisis óseas de las articulaciones diartroidales con la función de absorber impactos y contener el desgaste por rozamiento debido al movimiento. En el adulto es un tejido avascular, por lo que recibe los nutrientes del líquido sinovial y de los vasos sanguíneos del hueso subcondral. En su mayor parte está compuesto de una matriz extracelular altamente especializada compuesta de fibras de proteoglicanos y distintos tipos de colágeno, entre los cuales el más abundante (en torno a un 90%) es el colágeno de tipo II. Las fibras de colágeno se estructuran como una malla elástica retenedora de agua que, por ser un fluido incompresible, proporciona al cartílago rigidez y resistencia a la tracción y, al mismo tiempo, flexibilidad. Por otra parte, los condrocitos son las únicas células presentes en el cartílago. Son células diferenciadas y altamente especializadas en la síntesis y mantenimiento de los componentes de la matriz del cartílago como el colágeno de tipo II (Loeser, Goldring et al. 2012).

En el cartílago sano los condrocitos se encuentran en un estado cercano a la quiescencia, con poco recambio de matriz extracelular. Sin embargo, las articulaciones afectadas por OA sufren cambios fenotípicos en los condrocitos, que se hipertrofian y comienzan a producir y liberar mediadores que conducen a la calcificación del tejido. Se sabe que los condrocitos se activan principalmente a través del factor nuclear-κB (NF-κB) y las vías de proteín-quinasas activadas por mitógenos (MAPKs) (Goldring y Marcu 2009), que activan la expresión, entre otras, de ciclooxigenasa (COX)-2, prostaglandina E sintasa microsomal (mPGES)-1, sintasa de óxido nítrico inducible (iNOS), distintos receptores de citocinas y quimiocinas, así como de MMPs, una familia de endopeptidasas dependientes de zinc que tienen un papel fisiológico en la remodelación periódica de la matriz articular pero que, durante la activación osteoartrítica, producen una degradación proteica acelerada y anormal (Page-McCaw, Ewald et al. 2007).

Por otro lado, el tejido óseo también tiene un componente celular y estructural. La matriz tiene un importante componente proteico, principalmente en la forma de colágeno de tipo I y glicoproteínas, así como un componente mineral compuesto de cristales de hidroxiapatita en su mayor parte (Goldring y Goldring 2010). En el componente celular, por su parte, destacan los osteoblastos y los osteoclastos, cuya acción coordinada permite la remodelación fisiológica del hueso. Los osteoclastos permiten la reabsorción de la matriz, mientras que los osteoblastos producen la estructura que habrá de reemplazar a la anterior. Al final del proceso, la mayoría de osteoblastos mueren por apoptosis, otros

quedan quiescentes a la espera de nuevas remodelaciones, y una porción se diferencia en osteocitos residentes en el tejido (Khosla, Westendorf et al. 2008).

Pese a haberse definido tradicionalmente como patología de origen no inflamatorio, hoy se reconoce la relevancia de este componente en la OA que, por la presencia crónica de mediadores inflamatorios en el tejido, contribuye en gran medida a la progresión de la enfermedad. Entre los mediadores inflamatorios más relevantes en OA destacan las citocinas, las quimiocinas, otras moléculas como PGE_2, así como la producción de especies reactivas de oxígeno (ROS) y estrés oxidativo (Loeser 2013).

Las citocinas, por su parte, son mediadores centrales del sistema inmune que participan activamente en la degradación del tejido articular en la OA. Las más relevantes en esta patología son la IL-1β, el factor de necrosis tumoral (TNF)α y la IL-6 (Kapoor, Martel-Pelletier et al. 2011). Tanto IL-1β como TNFα son producidas por los condrocitos y osteoblastos, e inducen la producción de factores catabólicos. Asimismo, se sabe que inhiben la actividad anabólica de los condrocitos, previniendo la biosíntesis de los componentes principales de la matriz (Kobayashi, Squires et al. 2005). En cultivos celulares, el tratamiento con IL-1β inhibe la expresión de colágeno de tipo II y promueve la síntesis de MMPs, IL-6, iNOS, COX-2, mPGES-1 y muchos otros mediadores que agravan el estado inflamatorio (Clérigues, Guillén et al. 2012). Por su parte, la PGE_2, sintetizada por COX-2 y mPGES-1, es el mediador más implicado en la generación de dolor. Contribuye a la degradación articular estimulando la producción de MMPs, induciendo la apoptosis de condrocitos y promoviendo la reabsorción ósea (Haversath, Catelas et al. 2012). Al mismo tiempo, activa la expresión del ligando de receptor activador para el factor nuclear κB (RANKL) en osteoblastos, lo que favorece la diferenciación de los osteoclastos. Por último, PGE_2 puede estimular la producción de mediadores como IL-1β, retroalimentando el proceso inflamatorio que sufre la articulación (Li, Ellman et al. 2009).

IL-1β y TNFα también promueven la liberación de ROS e inhiben distintas enzimas antioxidantes como la superóxido dismutasa, la catalasa o la glutatión peroxidasa, generando un desequilibrio hacia los procesos oxidantes que conducen a un estado de estrés oxidativo, acelerando y agravando la degradación articular en la OA. Además, las ROS reaccionan descontroladamente con toda clase de proteínas, provocando su inactivación (Minguzzi, Cetrullo et al. 2018). De hecho, el estrés oxidativo altera el proceso de remodelado óseo, agravando el desequilibrio entre la actividad osteoclástica

y osteoblástica. En primer lugar, promueve la diferenciación de los pre-osteoclastos, reforzando la reabsorción de hueso. Pero las ROS inducen también la apoptosis de los osteoblastos y osteocitos a través de distintas vías de señalización, favoreciendo de nuevo la osteoclastogénesis (Domazetovic, Marcucci et al. 2017). Además, hay que destacar que los condrocitos son células altamente sensibles al estrés oxidativo debido a su entorno avascular y sus tasas metabólicas bajas. Las principales ROS que contribuyen al estrés oxidativo en estas células son el peroxinitrito y el peróxido de hidrógeno. La enzima iNOS se expresa tras la activación por citocinas y otros mediadores inflamatorios y promueve la producción excesiva de NO. La señalización por citocinas lleva a una producción exagerada de ROS, que empeoran la fisiología del condrocito y contribuyen a la degradación del cartílago (Henrotin, Kurz et al. 2005). Por otra parte, tanto en condrocitos como en osteoblastos, la mayoría de las ROS son producidas por la mitocondria como consecuencia de la fosforilación oxidativa, lo que genera un potencial de protones a través de la membrana interna mitocondrial. Estas especies pueden activar diferentes rutas de señalización oxidativas y dañar componentes de la mitocondria, alterando su función (Balaban, Nemoto et al. 2005).

El estrés oxidativo está muy relacionado con la senescencia celular, un estado celular no proliferativo asociado al envejecimiento y, por tanto, de gran relevancia en el contexto de la OA. Se sabe que los agentes causantes de estrés oxidativo pueden causar daño al DNA en condrocitos osteoartríticos, promoviendo la actividad β-galactosidasa asociada a senescencia (Platas, Guillén et al. 2016). Tradicionalmente, la senescencia se clasifica en senescencia replicativa, consecuencia de distintos procesos relacionados con el envejecimiento como el acortamiento telomérico; y senescencia asociada a estrés, como el estrés oxidativo producido por varios estados patológicos. En general, las células senescentes presentan cierta hipertrofia, disminuyen su actividad proliferativa y muestran ciertos marcadores específicos como el aumento de actividad β-galactosidasa, la acumulación de focos de heterocromatina en respuesta al daño genómico, un fenotipo hipersecretor y la sobreexpresión de p16, p21 y p53 (Carnero 2013).

La senescencia de los condrocitos provoca una incapacidad para reparar el cartílago, lo que favorece su degeneración. En el hueso, la acumulación de células senescentes interfiere en la osteogénesis, alterando el equilibrio homeostático del tejido. En ambos escenarios, la hiper-secreción de factores inflamatorios puede contribuir a retroalimentar el estado inflamatorio crónico y agravar la enfermedad, incrementando la intensidad del

remodelado óseo y degradación articular (Portal-Núñez, Esbrit et al. 2016). Por ello, el estudio de la senescencia celular en la OA como patología prevalente en población de edad avanzada cobra una doble importancia.

OBJETIVOS

En nuestro laboratorio hemos demostrado previamente los efectos antiinflamatorios, condroprotectores y antisenescentes del secretoma o medio acondicionado proveniente de células madre mesenquimales de tejido adiposo en células de la articulación osteoartrítica. Por lo tanto, en la presente tesis doctoral hemos pretendido profundizar en el conocimiento de la función y potencialidad terapéutica del secretoma en las células articulares.

Entre los componentes de este secretoma, las vesículas extracelulares destacan como posibles nuevas terapias biológicas heterólogas por su capacidad de señalización paracrina y su fácil administración y dosificación. Por ello, hemos planteado los siguientes objetivos:

1. Estudiar la morfología y composición de las vesículas extracelulares obtenidas a partir del medio acondicionado de células madre mesenquimales de tejido adiposo procedente de lipectomía reconstructiva de pacientes sanos no obesos.

2. Evaluar la capacidad antiinflamatoria de dichas vesículas en cultivos primarios de condrocitos osteoartríticos estimulados con IL-1β, comparativamente con el medio acondicionado del que proceden.

3. Determinar el papel en la senescencia celular de las vesículas extracelulares en cultivos primarios de osteoblastos osteoartríticos estimulados con IL-1β, en relación con el medio acondicionado del que proceden.

ARTÍCULOS DE INVESTIGACIÓN

CAPÍTULO 1

Microvesicles from human adipose tissue-derived mesenchymal stem cells as a new protective strategy in osteoarthritic chondrocytes

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Cellular Physiology and Biochemistry (2018) **47**:11-25.

Microvesicles from human adipose tissue-derived mesenchymal stem cells as a new protective strategy in osteoarthritic chondrocytes

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Running headline: Chondroprotective actions of AD-MSC MV

Keywords: extracellular vesicles, adipose tissue-derived mesenchymal stem cells, chondrocyte, inflammation, osteoarthritis.

Abstract

***Background/Aims*:** Chronic inflammation contributes to cartilage degeneration during the progression of osteoarthritis (OA). Adipose tissue-derived mesenchymal stem cells (AD-MSC) show great potential to treat inflammatory AD-MSC and degradative processes in OA and have demonstrated paracrine effects in chondrocytes. In the present work, we have isolated and characterized the extracellular vesicles from human AD-MSC to investigate their role in the chondroprotective actions of these cells.

Methods: AD-MSC were isolated by collagenase treatment from adipose tissue from healthy individuals subjected to abdominal lipectomy surgery. Microvesicles and exosomes were obtained from conditioned medium by filtration and differential centrifugation. Chondrocytes from OA patients were used in primary culture and stimulated with 10 ng/ml interleukin(IL)-1β in the presence or absence of AD-MSC microvesicles, exosomes or conditioned medium. Protein expression was investigated by ELISA and immunofluorescence, transcription factor-DNA binding by ELISA, gene expression by real-time PCR, prostaglandin E_2 (PGE_2) by radioimmunoassay, and matrix metalloproteinase (MMP) activity and nitric oxide (NO) production by fluorometry.

***Results*:** In OA chondrocytes stimulated with IL-1β, microvesicles and exosomes reduced the production of inflammatory mediators tumor necrosis factor-α, IL-6, PGE_2 and NO. The downregulation of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 would lead to the decreased PGE_2 production while the effect on NO could depend on the reduction of inducible nitric oxide synthase expression. Treatment of OA chondrocytes with extracellular vesicles also decreased the release of MMP activity and MMP-13 expression whereas the production of the anti-inflammatory cytokine IL-10 and the expression of collagen II were significantly enhanced. The reduction of inflammatory and catabolic mediators could be the consequence of a lower activation of nuclear factor-κB and activator protein-1. The upregulation of annexin A1 specially in MV may contribute to the anti-inflammatory and chondroprotective effects of AD-MSC.

***Conclusion*:** Our data support the interest of AD-MSC extracellular vesicles to develop new therapeutic approaches in joint conditions.

Introduction

Chronic production of inflammatory mediators has important implications for chondrocyte metabolism in joint diseases. Pro-inflammatory cytokines contribute to alterations in osteoarthritis (OA) joint metabolism through the production of inflammatory mediators and catabolic enzymes capable of destroying cartilage matrix [1-3], and the downregulation of anti-inflammatory and anabolic genes [4]. OA remains a leading cause of disability in the elderly without an effective treatment. At present, the treatment of OA is targeted to control symptoms although innovative therapeutic approaches such as joint injection of mesenchymal stem cells (MSC) and differentiation into chondrocytes using appropriate scaffolds to regenerate cartilage are being investigated [5].

MSC have opened a new avenue for treating tissue injury and inflammation. In particular, adipose-derived mesenchymal stem cells (AD-MSC) show great therapeutic potential and have demonstrated protective properties in animal models of OA. Therefore, injection of these cells into the knee joint is able to reduce inflammation and cartilage degradation induced by collagenase in mice [6] or by anterior cruciate ligament transection in rabbits [7].

MSC cell therapy has demonstrated beneficial effects despite short-lived survival of the delivered cells suggesting that secreted factors may be the active components. There is evidence that MSC secrete into their microenvironment a number of cytokines and growth factors that regulate intracellular signaling pathways in neighboring cells, promote angiogenesis and recruitment of stem/progenitor cells, or exert trophic and immunomodulatory effects (reviewed in [8]). As a result, treatment of OA chondrocytes or synovial cells with conditioned medium (CM) from bone marrow MSC or AD-MSC in an inflammatory environment can inhibit the production of inflammatory and catabolic agents [9, 10].

It is now recognized that MSC release extracellular vesicles (EV) as vehicles for intercellular communication. In particular, microvesicles (MV) are a heterogeneous population of spherical structures with a diameter of 100–1000 nm which are released by ectocytosis of the plasma membrane [11] while exosomes (EX) are membrane vesicles with a diameter of 40–100 nm, formed by endocytosis, stored intracellularly and secreted when endosomal structures fuse with the plasma membrane [12]. In recent years, there has been significant interest in MSC EV as mediators of regenerative responses with

potential therapeutic applications in cardiovascular diseases [13], rheumatic diseases [14], fracture healing [15], neurodegeneration [16] or immunomodulation [17]. In relation with cartilage metabolism, it has been shown recently that EX from HuES9 human embryonic stem cells are able to repair osteochondral defects in rats [18] and EX from miR-140-5p-overexpressing human synovial MSC prevent the development of OA-like changes after surgical destabilization of the rat knee [19].

We have previously reported the anti-inflammatory and protective properties of CM from AD-MSC in OA chondrocytes [20, 21]. Despite these studies, the possible contribution of EV to the observed effects is not known. To address this issue, we have assessed how MV and EX isolated from this CM could affect the metabolism of OA chondrocytes by modulating inflammatory and degradative pathways relevant in joint destruction.

Materials and methods

Adipose-derived mesenchymal stem cells

AD-MSC were obtained from the adipose tissue of 11 non-obese donors (4 men and 7 women, aged 53.8±7.4 years, mean±SEM) who had undergone abdominoplasty. The experimental design was approved by the Institutional Ethical Committees (University of Valencia and La Fe Polytechnic University Hospital, Valencia, Spain). Samples were obtained from donors after they provided informed consent according to the Helsinki Declaration of 1975, as revised in 2013.

Adipose tissue samples were washed with phosphate-buffered saline (PBS), minced, digested at 37ºC for 1 h with 2% of type I collagenase (Gibco, Life Technologies, Madrid, Spain) and filtered through a 100 μm cell strainer (BD Biosciences, Bedford, MA, USA). Then, cells were washed with DMEM/HAM F12 (Sigma-Aldrich, St. Louis, MO, USA) containing penicillin and streptomycin (1%), seeded onto tissue culture flasks ($1–2×10^6$ cells/ml, 30 ml culture) in DMEM/HAM F12 medium with penicillin and streptomycin (1%) supplemented with 15% EV-free human serum, and incubated at 37ºC in a 5% CO_2 atmosphere. Human serum was obtained from whole-blood donations of AB-blood-group-typed donors according to the criteria of Valencia Transfusion Centre. To eliminate the EV fraction, serum was centrifuged during 18 h at 120,000×g and 4ºC using a SW-28 swinging-bucket rotor (Beckman Coulter, Brea, CA, USA). At 24 h, when cells reached

semiconfluence, culture plates were washed and the AD-MSC phenotype confirmed by flow cytometry (Flow Cytometer II, BD Biosciences, San Jose, CA, USA) using specific antibodies: anti-CD105-PE, antiCD90PerCP-eFluo 710, anti-CD34APC (eBioscience, Inc., San Diego, CA, USA), and anti-CD45-PE (BD Pharmingen™, BD Biosciences). Cellular viability was determined with propidium iodide.

Immortalized Human Keratinocytes (HaCaT) cell line was kindly provided by Prof. Norbert E. Fusenig (German Cancer Research Institute, Heidelberg, Germany). Cells were cultured in DMEM/HAM F12 with penicillin and streptomycin (1%) supplemented with 15% EV-free human serum, and incubated at 37ºC in a 5% CO_2 atmosphere.

CM was collected from AD-MSC and HaCaT culture cells at passage 0 every 48 h of culture. It was pooled, centrifuged, and stored in sterile conditions at −80º C prior to further use.

Isolation of EV

EV were obtained from the CM of AD-MSC and HaCaT cells using a filtration/centrifugation-based protocol. Cellular debris was eliminated by pelleting with centrifugation at 300×g for 10 min at 4°C. EV were then collected from the supernatant through differential centrifugation steps. Briefly, CM was filtered through 800 nm filter (Merck, Darmstadt, Germany) and centrifuged at 12,200×g for 20 min at 4°C to pellet MV. Then, supernatants were filtered through 200 nm filter (Merck, Darmstadt, Germany) and centrifuged at 100,000×g for 90 min at 4°C. The resulting pellets containing EX were washed once with sterile PBS, resuspended in 15 µl of PBS and stored at -80ºC until use.

Tunable resistive pulse sensing (TRPS)

EV preparations were analyzed by TRPS using a qNano instrument (IZON Sciences Ltd., Oxford, UK) [22]. NP100, and NP300 nanopore membranes were used to measure the samples of EX and MV, respectively. At least 500 events/sample were counted. Calibration was performed using calibration beads SKP200 and SKP400, provided by the manufacturer.

Transmission electron microscopy (TEM)

EV preparation for TEM was performed by the Microscopy Service (SCSIE, University of Valencia). Briefly, LR-white resin inclusion was performed fixing EV samples with

Karnovsky fixative, inclusion in agar, followed by water washing and dehydration in 30% EtOH, 50% EtOH, 70% EtOH and 96% EtOH. Finally, samples were sequentially incubated for 2 h in 33% LR-white resin in 96% EtOH, 66% LR-white resin in 96% EtOH, 66% LR-white resin in 100% EtOH and 100% LR-white resin in 100% EtOH. Samples were filtered in resin and polymerized at 60ºC for 48 h. Then, ultrathin slices (60 nm) were made with a diamond blade (DIATOME, Hartfield, USA) in eyelet grilles in a UC6 Ultracut (Leica, Wetzlar, Germany) and stained with uranyl acetate 2% for 25 min and lead citrate 3% for another 12 min prior to visualization in Jeol-1010 (JEOL Ltd. Tokyo, Japan) at 60 kV. Images were acquired with a digital camera MegaView III with Olympus Image Analysis Software (Olympus, Tokyo, Japan).

For gold-immunostaining, mouse anti-human CD63 monoclonal and goat anti-mouse IgG H&L (10 nm Gold) polyclonal antibodies from Abcam (Cambridge, MA, USA) were used. EV were fixed with Karnovsky's fixative and then processed in resin as described above. Grids containing the samples were blocked with PBS/0.8% bovine serum albumin (BSA)/0.1% gelatin, and 2 µl of primary antibody in PBS/0.5% BSA were added. Grids were then washed with PBS/0.5% BSA, incubated with the gold-labeled secondary antibody in PBS/0.5% BSA for 30 min, and then washed in 100 µl drops of PBS/0.5% BSA. Control grids incubated with only secondary antibodies were also used. The grids were stained with 2% uranyl acetate and then viewed for TEM using a Jeol JEM1010 microscope at 60 kV and images were acquired with a digital camera MegaView III with Olympus Image Analysis Software.

Flow Cytometry

Annexin V positive MV were determined with the FITC Annexin V Detection Kit I (BD Biosciences). Immediately before incubation, antibody was ultracentrifuged and washed in 0.2 µm filtered-PBS to avoid noise, and all solutions were previously filtered. For labeling, EV were diluted down to 500,000 particles/ml in labeling buffer with FITC-conjugated annexin V and incubated at room temperature for 1h in dark. After labeling, EV were twice washed and recovered in filtered PBS. EV were then analyzed at a flow rate on a LSR Fortessa X-20 flow cytometer (BD Biosciences) and data registered with the software DIVA 8.0 processed with the software FlowJo (FlowJo LLC, Ashland, OR, USA). The gating window for counting EV and discriminating against background noise was set using forward and side scatter plots for Megamix-Plus FSC fluorescent beads (BioCytex, Marseille, France) of diameters 100 nm, 300 nm, 500 nm and 900 nm, and

FITC fluorescent positivity established as compared to unlabeled EV and EV-free annexin V and FITC-antibody solutions.

OA chondrocytes

Knee specimens were obtained from patients diagnosed with advanced OA (27 women and 14 men, aged 65.6±12.0 years, mean±SEM) who had undergone total joint replacement. The experimental design was approved by the Institutional Ethical Committees as indicated above. Cartilage was dissected from the femoral condyles and tibial plateau of the knee joint and diced into small pieces. Human articular chondrocytes were isolated by sequential enzymatic digestion: 1 h with 0.1 mg/ml hyaluronidase (Sigma-Aldrich) followed by 12–15 h with 2 mg/ml type IA collagenase (Sigma-Aldrich) in DMEM/HAM F12 containing penicillin and streptomycin (1%) at 37ºC in 5% CO_2 atmosphere. The digested tissue was filtered through a 70 μm nylon mesh (BD Biosciences), washed, and centrifuged. Cell viability was greater than 95% according to the Trypan blue exclusion test. All experiments were performed with chondrocyte primary cultures at semiconfluence (270×10^3 cells/well in 6-well plates or 1.5×10^6 cells in 3.5 cm plates). Chondrocytes were maintained with 5% CO_2 at 37ºC in DMEM/HAM F12 containing penicillin and streptomycin (1%), supplemented with 10% fetal bovine serum (Sigma-Aldrich).

To perform the experiments, chondrocytes and explants were incubated for different times in DMEM/HAM F12 containing penicillin and streptomycin (1%) supplemented with 15% EV-free human serum and stimulated with interleukin(IL)-1β (10 ng/ml) in the presence or absence of AD-MSC- or HaCaT MV ($3.6x10^7$ particles/ml), EX ($7.2x10^7$ particles/ml) or CM (0.4 ml for 24-well plates, 1 ml for 6-well plates or 1.5 ml for 3.5 cm plates).

For explant cultures, full-thickness pieces of cartilage were removed from the femoral condyles. Slices measuring ~2 mm in width × 2 mm in length were dissected from the tissue. Explants were transferred to 24-well plates (10 explants/well) containing DMEM/HAM F12 medium supplemented with penicillin and streptomycin (1%), and 10% fetal bovine serum, and they were incubated in a humidified 5% CO_2 incubator at 37°C for 2 days before assays to allow them to stabilize.

MTT Assay

The mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) to formazan was assayed in OA chondrocytes stimulated with IL-1β (10 ng/ml) and treated with MV ($3.6x10^7$ particles/ml), EX ($7.2x10^7$ particles/ml) or CM (0.4 ml) in 24-well plates for 24 h. Cells were then incubated with MTT (200 μg/ml) for 2 h. Medium was removed and cells were solubilized in dimethyl sulfoxide (100 μl) to quantitate formazan at 550 nm using a Victor3 microplate reader (PerkinElmer España, Madrid, Spain).

Proteomic analysis of EV by mass spectrometry

Proteomic characterization of EV samples was performed by the Proteomics Service of the Servei Central de Suport a la Investigació Experimental (Universitat de València). Briefly, 10 μg per EV sample were digested with Triton X-100 and separated by 1-D SDS PAGE. Peptides were generated by a trypsin digestion, extracted, and examined by LC using a NanoLC Ultra 1-D plus Exsigent (Eksigent Technologies, Dublin, CA, USA) which was directly connected to an AB SCIEX TripleTOF 5600 mass spectrometer (AB SCIEX, Framingham, MA, USA) in direct injection mode. After LC-MS/MS, the SCIEX.wiff data-files were processed using ProteinPilot v5.0 search engine (AB SCIEX). The resulting ProteinPilot group file was loaded into PeakView® (v2.1, AB SCIEX) and peaks were extracted with a peptide confidence threshold of 99% confidence (Unused Score ≥ 1.3) and a false discovery rate (FDR) lower than 1%. For this, the MS/MS spectra of the assigned peptides were extracted by ProteinPilot, and only the proteins that fulfilled the following criteria were validated: (1) peptide mass tolerance lower than 10 ppm, (2) 99% of confidence level in peptide identification, and (3) complete b/y ions series found in the MS/MS spectrum. The identified proteins were quantified using PeakView® from normalized label-free quantification (LFQ) intensity data. The quantitative data obtained by PeakView® were analyzed using MarkerView® (v1.2, AB SCIEX). First, areas were normalized by total areas summa. Principal Component Analysis (PCA) was performed to evaluate the discriminative ability of proteins in different EV fractions. Bioinformatics analysis of identified and validated SP-proteins was manually performed using the comprehensive bioinformatics tool for functional annotation UniProt KB database (www.uniprot.org) in combination with PANTHER (www.pantherdb.org) and FunRich (http://www.funrich.org). Data are available via ProteomeXchange with identifier PXD009077 and 10.6019/PXD009077.

ELISA

Chondrocytes were stimulated with IL-1β (10 ng/ml) in presence or absence of MV ($3.6x10^7$ particles/ml), EX ($7.2x10^7$ particles/ml) or CM (1 ml) for 24 h, or 1h for transcription factors detection. Supernatants were centrifuged and stored at -80ºC until analysis. Tumor necrosis factor-α (TNFα), IL-6, and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA) kits from eBioscience (San Diego, CA, USA) with a sensitivity of 4.0 pg/ml for TNFα and IL-6, and 2.0 pg/ml for IL-10. Nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) binding to DNA was quantified by ELISA in nuclear extracts using the Nuclear Extract Kit Active Motif for nuclei extraction followed by TransAM p65 NF-κB and TransAM c-Jun Activation Assay kits (Active Motif Europe, Rixensart, Belgium), according to the manufacturer's recommendations.

Determination of MMP activity, NO and PGE_2

Chondrocytes were stimulated as indicated above and supernatants were harvested and centrifuged. For matrix metalloproteinase (MMP) activity determination, supernatants were incubated with p-aminophenylmercuric acetate for 12 h at 37ºC to activate MMPs. Then, supernatants were transferred to a 96-well plate. After addition of the 5- FAM peptide substrate (AnaSpec Inc., San Jose, CA, USA), fluorescence was measured at 490 nm (excitation)/520 nm (emission) in a Victor3 microplate reader (PerkinElmer España). Prostaglandin E_2 (PGE_2) was quantitated in supernatants by radioimmunoassay [23] and nitric oxide (NO) production was assessed by fluorometric determination of nitrite levels [24] using a Victor3 microplate reader (PerkinElmer España).

Real-time PCR

Total RNA was extracted from OA chondrocytes using the TriPure reagent (Roche Life Science, Barcelona, Spain) according to the manufacturer's instructions. Reverse transcription was accomplished on 1 μg of total RNA using random primers and Transcriptor First Strand cDNA Synthesis Kit (Roche Life Science). PCR assays were performed in duplicate on an iCycler Real-Time PCR Detection System using SYBR Green PCR Master Mix (Bio-Rad Laboratories, Richmond, CA, USA). Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany) [20]. For each sample, differences in threshold cycle (ΔCt) values were calculated by correcting the Ct of the gene of interest to the Ct of the reference gene β-actin. Relative gene expression was expressed as $2^{-\Delta\Delta Ct}$ with respect to non-stimulated cells.

Immunofluorescence

Chondrocytes were seeded at $20x10^3$ cells/well in Lab-tek chambers (Thermo Scientific, Rochester, NY, USA) and stimulated with IL-1β (10 ng/ml) in the presence or absence of MV ($3.6x10^7$ particles/ml), EX ($7.2x10^7$ particles/ml) or CM (0.2 ml) for 24h (annexin A1) or 5 days (collagen II). Cells were fixed with 4% formaldehyde in PBS for 30 min at 4ºC, blocked with 1% BSA in PBS for 20 min at room temperature and incubated with rabbit anti-human type II collagen polyclonal antibody (Chemicon/Millipore, Schwalbach, Germany) or MaxPab rabbit anti-annexin A1 polyclonal antibody (Abnova, New Taipei, Taiwan) followed by incubation with goat anti-rabbit IgG-FITC (R&D Biosystems, Abingdon, UK). Slides were mounted in Prolong Gold antifade reagent with DAPI (Molecular Probes, Invitrogen, Life Technologies) and examined under a confocal microscope (Olympus FV1000, Tokyo, Japan). Collagen II- or annexin A1-positive cells were observed in 6 microscopic fields of each well. Fluorescence density was quantified using ImageJ software (National Institutes of Health, USA).

Annexin A1 blocking

MV were incubated with MaxPab rabbit anti-ANXA1 polyclonal antibody (Abnova, New Taipei, Taiwan) at 20 μg/ml for 1 h at 4ºC, then washed with PBS and pelleted at 12.600×g. Chondrocytes were stimulated with IL-1β (10 ng/ml) in presence or absence of MV ($3.6x10^7$ particles/ml) previously treated with anti-annexin A1 antibody or MV control for 24 h. IL-6 was determined in supernatants by ELISA and collagen type-II in chondrocytes by immunofluorescence as indicated above.

Statistical analysis

Data are expressed as mean and standard error of the mean (mean ± SEM). Data were analyzed by one-way analysis of variance (ANOVA) followed by the Sidak's post test using the GraphPad Prism 7.0 software (Graph Pad Software, La Jolla, CA, USA). A *P* value of less than 0.05 was considered statistically significant.

Results

Characterization of EV and effect on cell viability

MV and EX fractions were isolated from AD-MSC CM as indicated in Materials and methods. TRPS analysis indicated a mean concentration of MV and EX of $8.0x10^9$ and $3.8x10^{10}$ particles/ml, respectively. In addition, we isolated MV and EX from HaCaT cells as a negative control in functional studies. The MV fraction had an average size of 279±94 nm and a concentration of $6.5x10^{10}$ particles/ml while the EX fraction had an average size of 104±19 nm and a concentration of $1.1x10^{12}$ particles/ml.

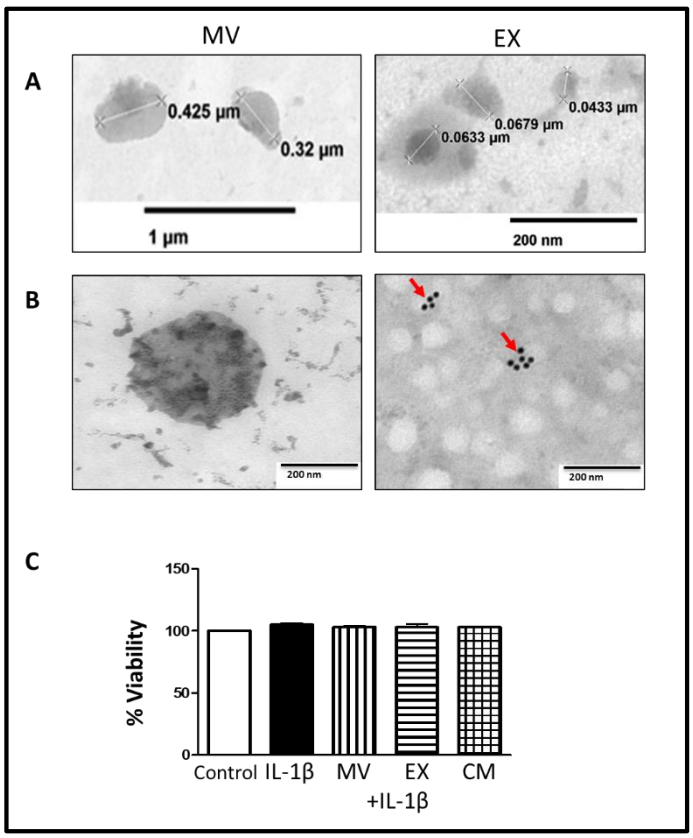

Fig. 1 Characterization of MV and EX isolated from CM from AD-MSC and effect on cell viability. Representative transmission electron microscopy images of MV and EX with estimated size (A). Immunostaining with gold-labeled anti-CD63 antibodies (B). Red arrows show positive staining. C: Viability (%) of OA chondrocytes in the presence of IL-1β and AD-MSC EV, determined by the MTT method. Results are expressed as mean±SEM from 3 separate experiments with cells from separate donors.

Representative TEM images of MV and EX from AD-MSC with estimated size are shown in Figure 1A. Immunostaining with gold-labeled anti-CD63 antibodies was performed and we observed the presence of CD63 labeling in EX (Figure 1B). In addition, 56.7±16.4 % (n=3) of annexin V positive MV were detected by flow cytometry. To assess the effects of EV on OA chondrocytes, we selected the concentrations of $3.6x10^7$ particles/ml for MV and $7.2x10^7$ particles/ml for EX which are in the range of concentrations present in CM used in the same experiments. We confirmed that MV and EX at these concentrations do not affect cell viability by the MTT method (Figure 1C).

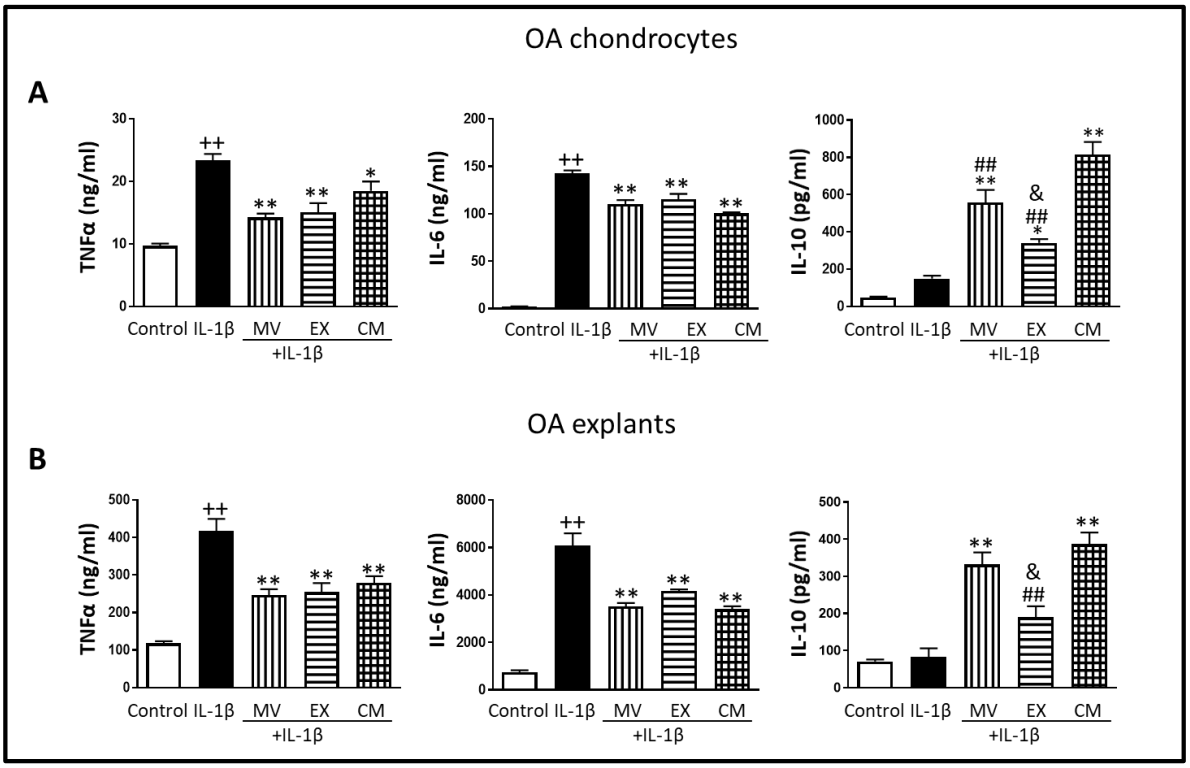

Fig. 2 Effects of EV from AD-MSC on cytokine release by OA chondrocytes (A) and explants (B). IL-6, TNFα and IL-10 were measured by ELISA in culture supernatants. Cultures were treated with IL-1β alone or in combination with EV or CM from AD-MSC for 24 h. Results are expressed as mean±SEM from 3 separate experiments with cells from separate donors. ++P<0.01 compared to control (non-stimulated cells); *P<0.05, **P<0.01 compared to IL-1β; ## P<0.01 compared to CM; &P<0.05 compared to MV.

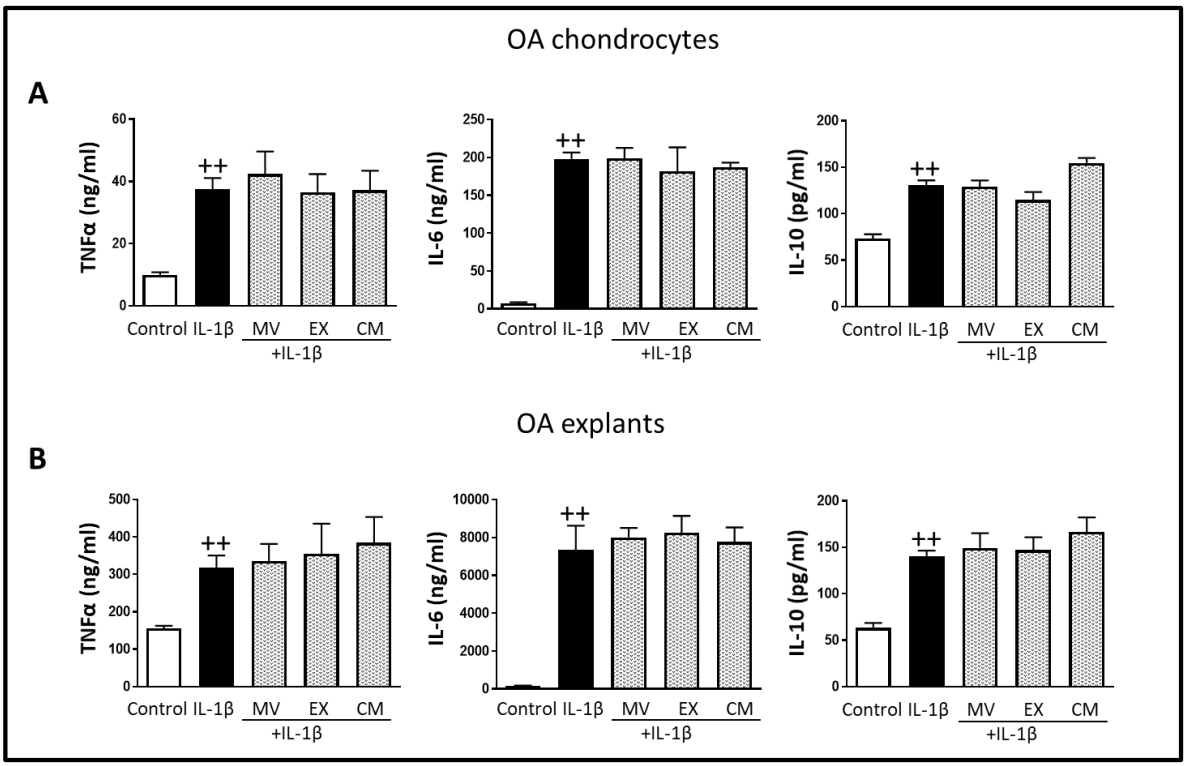

Fig. 3 Effects of EV and CM from HaCaT cells on cytokine release by OA chondrocytes (A) and explants (B). IL-6, TNFα and IL-10 were measured by ELISA in culture supernatants. Cultures were treated with IL-1β alone or in combination with EV or CM from HaCaT cells for 24 h. Results are expressed as mean±SEM from 3 separate experiments with cells from separate donors. ++P<0.01 compared to control (non-stimulated cells).

Effects on cytokines

To study how the production of relevant inflammatory and catabolic mediators was modulated by AD-MSC-derived EV, OA chondrocytes were incubated with IL-1β in the presence or absence of MV, EX or CM. Stimulation of OA chondrocytes with IL-1β for 24 h increased the release of the pro-inflammatory cytokines IL-6 and TNFα into the culture medium compared with control non-stimulated cells (Figure 2A). Treatment with MV, EX or CM significantly reduced the levels of both pro-inflammatory cytokines. In contrast, the release of the anti-inflammatory cytokine IL-10 was significantly enhanced when cells were treated with MV, EX or CM compared with cells treated with IL-1β alone. The highest effect was exhibited by CM followed by MV. In addition, we determined the effects of AD-MSC EV on cytokine production by OA explants which represent a more physiological setting for chondrocytes. As shown in Figure 2B, the behavior of MV, EX and CM in OA explants was very similar to that observed in OA chondrocytes. In order to determine if these effects of MV and EX are specific to EV from AD-MSC, we performed the same experiments in OA chondrocytes and OA explants but using EV and CM from HaCaT cells instead of AD-MSC. Figure 3A and B shows that MV, EX and CM from HaCaT cells were completely ineffective on cytokine production by OA chondrocytes or explants.

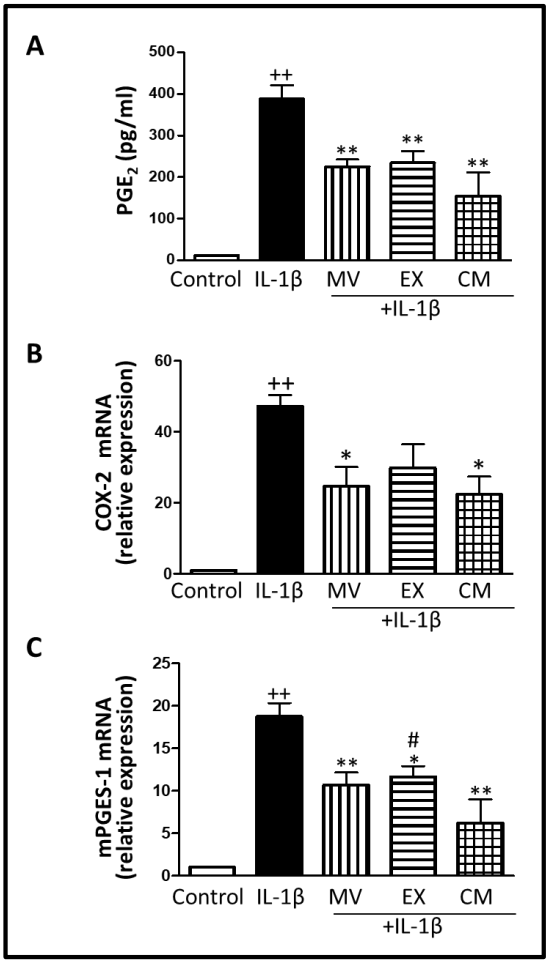

Fig. 4 PGE2 levels and COX-2 and mPGES-1 gene expression in OA chondrocytes. A: PGE2 levels were measured by radioimmunoassay in cell culture supernatants. B: COX-2 and C: mPGES-1 mRNA expression was determined by real-time PCR as indicated in materials and methods. Cultures were treated with IL-1β alone or in combination with EV or CM from AD-MSC for 24 h (mean±SEM from 5 separate experiments with cells from separate donors). ++P<0.01 compared to control (non-stimulated cells); *P<0.05, **P<0.01 compared to IL-1β; # P<0.05 compared to CM.

Effects on PGE_2 production and COX-2 and mPGES-1 expression

The levels of PGE_2 released into the culture medium of OA chondrocytes were determined to assess if this eicosanoid could be regulated by EV. As shown in Figure 4A,

PGE_2 levels in IL-1β-stimulated cells were significantly decreased by all treatments. IL-1β upregulates cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) which act in a coordinated manner to synthesize high levels of PGE_2 during inflammatory responses [25]. We determined the mRNA expression of COX-2 (Figure 4B) and mPGES-1 (Figure 4C) in OA chondrocytes which was reduced by treatment with EV or CM.

Effects on NO production and iNOS expression

IL-1β stimulation of OA chondrocytes results in upregulation of inducible nitric oxide synthase (iNOS) and NO production which was estimated by the levels of nitrite present in the culture medium (Figure 5A). We observed that MV, EX and CM significantly decreased the levels of nitrite in the medium and the mRNA expression of iNOS in OA chondrocytes compared with cells treated with IL-1β alone.

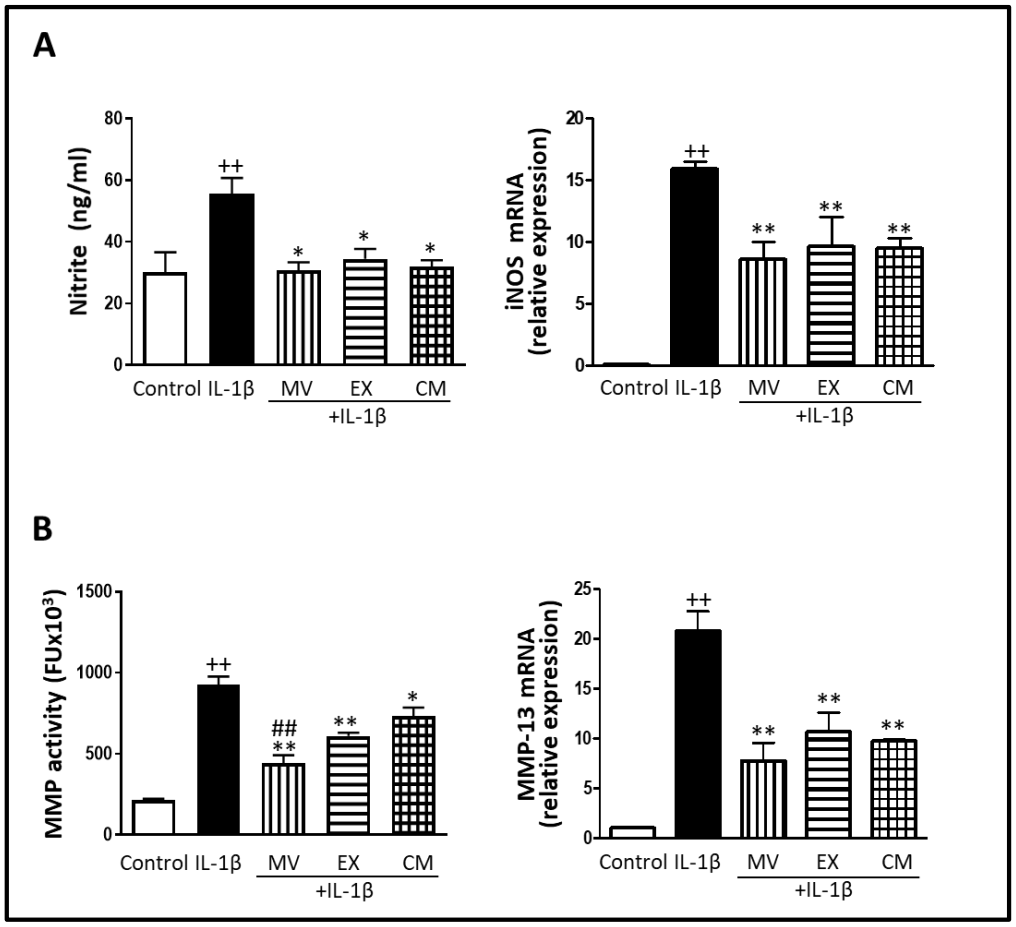

Fig. 5 NO production and iNOS gene expression (A), MMP activity and MMP-13 gene expression (B) in OA chondrocytes. Nitrite levels and MMP activity were measured by fluorometry in cell culture supernatants. iNOS and MMP-13 mRNA expression was determined by real-time PCR as indicated in materials and methods. Cultures were treated with IL-1β alone or in combination with EV or CM from AD-MSC for 24 h (mean±SEM from 4 (A) or 3 (B) separate experiments with cells from separate donors). FU, fluorescence units. ++P<0.01 compared to control (non-stimulated cells); *P<0.05, **P<0.01 compared to IL-1β; ## P<0.01 compared to CM.

Effects on MMPs

In order to evaluate the effects of treatments on total MMP activity, a fluorometric assay was performed as indicated in Materials and methods. Figure 5B shows that OA chondrocyte stimulation with IL-1β resulted in increased MMP activity in the culture medium. All treatments significantly reduced this activity and MV exerted a stronger effect compared with CM. MMP-13 (collagenase 3) has been implicated in the early phase of chondrocyte-mediated cartilage collagen breakdown [26]. A consistent induction of MMP-13 gene expression was seen following IL-1β stimulation of OA chondrocytes whereas treatment with MV, EX or CM significantly decreased it.

Effects on collagen II expression

It is known that IL-1β suppress the transcription of the chondrocyte-specific marker collagen type II [4] and induces its degradation [27]. Figure 6 shows that when IL-1β and EV or CM were present in the culture medium, these treatments significantly relieved the IL-1β-induced suppression of chondrocyte-specific collagen type II expression. Interestingly, MV were significantly more effective than EX or CM leading to a level of collagen II close to that of control chondrocytes (non-stimulated cells).

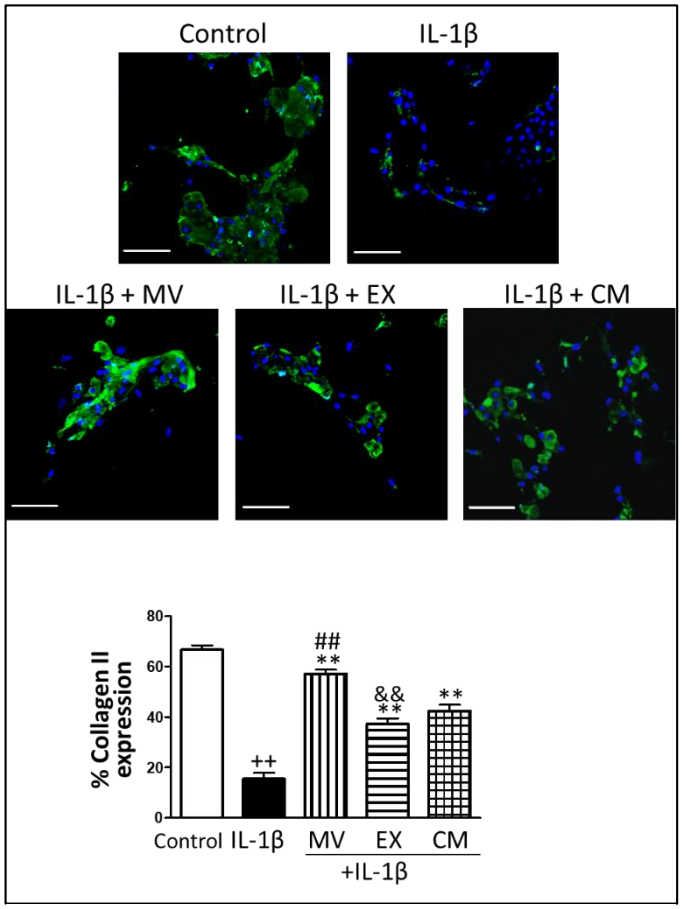

Fig. 6 Collagen II expression in OA chondrocytes. Collagen II protein expression was determined by immunofluorescence. Cultures were treated with IL-1β alone or in combination with EV or CM from AD-MSC for 5 days. Results are expressed as mean±SEM from 4 separate experiments with cells from separate donors. ++P<0.01 compared to control (non-stimulated cells); **P<0.01 compared to IL-1β; ## P<0.01 compared to CM; &&P<0.01 compared to MV. Bar= 50 µm.

Effects on transcription factors

The transcription factor NF-κB mediates many of the downstream

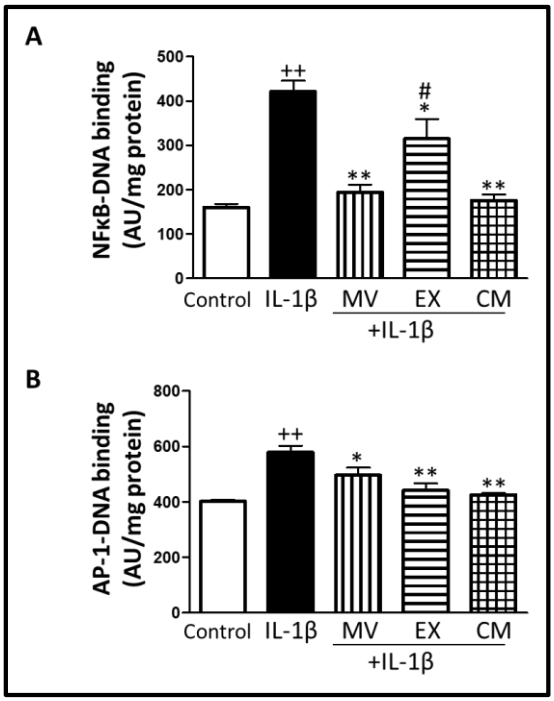

Fig. 7 Activation of transcription factors in OA chondrocytes. P65 NF-κB (A) and c-jun AP-1 (B) binding to DNA was measured by ELISA and expressed as arbitrary units (AU) per mg of protein. Cultures were treated with IL-1β alone or in combination with EV or CM from AD-MSC for 1 h. Results are expressed as mean±SEM from 4 separate experiments with cells from separate donors. ++P<0.01 compared to control (non-stimulated cells); *P<0.05, **P<0.01 compared to IL-1β; # P<0.05 compared to CM.

effects of IL-1β activating the transcription of pro-inflammatory and catabolic molecules. We have studied the effects of MV, EX and CM on the binding of p65 to DNA in the nucleus of OA chondrocytes stimulated with IL-1β. There was a marked enhancement of p65-DNA binding by this cytokine (Figure 7A) which was significantly reduced by MV, CM and to a lesser extent by EX. AP-1 also plays an important role in MMP transcription induced by cytokines [1]. DNA binding of c-Jun AP-1 was significantly activated by IL-1β (Figure 7B) while treatment with MV, EX or CM significantly decreased this process.

Proteomic analysis of AD-MSC EV

We performed a proteomic analysis of AD-MSC EV in order to identify their protein content and possible active components. 365 proteins were found in MV preparations, 217 in EX and 213 in CM. Figure 8A shows the Venn diagram indicating that 116 proteins were identified in MV, EX and CM. MV had a higher number of unshared proteins compared with EX (171 vs. 38).

These proteins are involved in different cellular process, mainly cellular growth and/or maintenance, immune response, protein metabolism and transport (Figure 8B). When compared with CM proteome, 42 unique proteins were identified in MV with a *P* value <0.01. Of them, 15 (Table 1) were over-represented with a fold-change of at least 2.5. Among EX proteins, 28 were found significantly different from the CM proteome, but only 3 were over-represented with a fold change of at least 2.5.

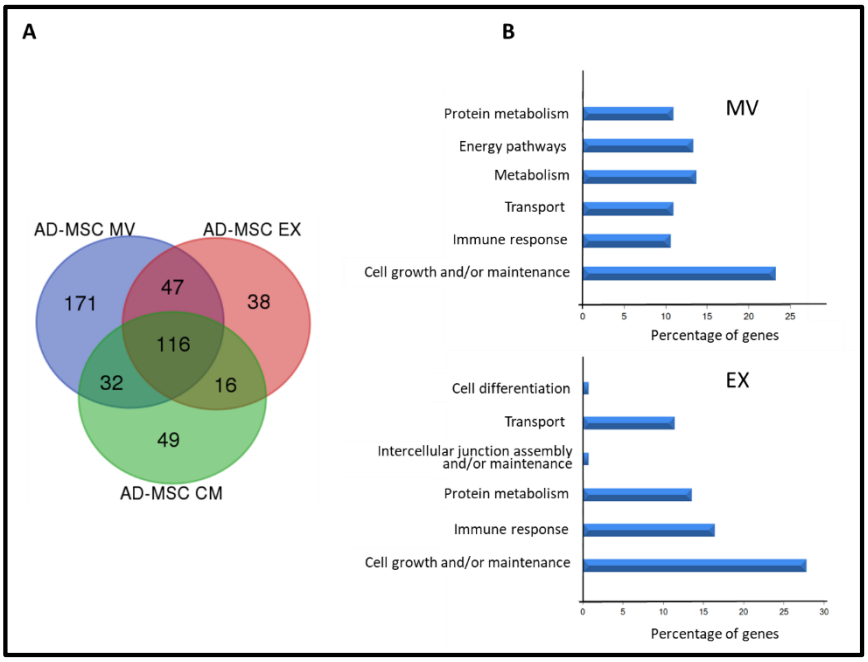

Fig. 8 Proteome characterization of EV from AD-MSC. A: Venn diagram. The number present in the circle represents the total number of identified proteins in particular data sets. B: Most over-represented biological processes (gene ontology terms) present in EV proteomes.

Table 1 Over-represented proteins in AD-MSC EV compared to CM.

Identified protein	Uniprot ID	Main function
MV		
Annexin A1	P04083	Immunomodulation
Apolipoprotein H	P02749	Negtive substrate binding
Butyryl-Cholinesterase	P06276	Esterase activity
CD81	P60033	Endocytic trafficking
Dermokine	Q6E0U4	Keratinocyte differentiation
Fermitin family homolog 3	Q86UX7	Integrin activation
Integrin β1	P05556	Cell-matrix collagen binding
Peroxiredoxin 6	P30041	Peroxidase/Phospholipase A2
Phosphoglycerate mutase 1	P18669	Canonical glycolysis
Junction Plakoglobin	P14923	α-catenin binding
Rab GDP dissociation inhibitor	P50395	Rab regulation
Tropomyosin 1	P09493	Actin binding
Tropomyosin 3	P06753	Actin binding
α-Actinin 4	O43707	Actin binding
α-Enolase	P06733	Canonical glycolysis
EX		
Carboxypeptidase N	P15169	Inflammatory peptides degradation
HSP70	P11142	Molecular chaperone
Pregnancy-zone protein	F5GXY0	Endopeptidase inhibitor

Effects on annexin A1

We have studied CM and EV effects on the expression of annexin A1. This protein is over-represented in MV (Table 1) and has demonstrated immunomodulatory and anti-inflammatory properties in different systems (reviewed in [28]). Fig. 9A shows that annexin A1 expression was significantly enhanced by treatment of chondrocytes with all fractions and mainly with MV. To explore the possibility that annexin A1 may contribute to the observed anti-inflammatory and chondroprotective effects, we neutralized this protein in the fraction showing the highest expression using a specific antibody and determined the consequences on the production of the inflammatory cytokine IL-6 and the expression of collagen II in OA chondrocytes in the presence of IL-1β. Annexin A1 blockade significantly reverted the inhibitory effects of MV on the inflammatory cytokine IL-6 (Fig. 9B) and the enhancement of type II collagen (Fig. 10).

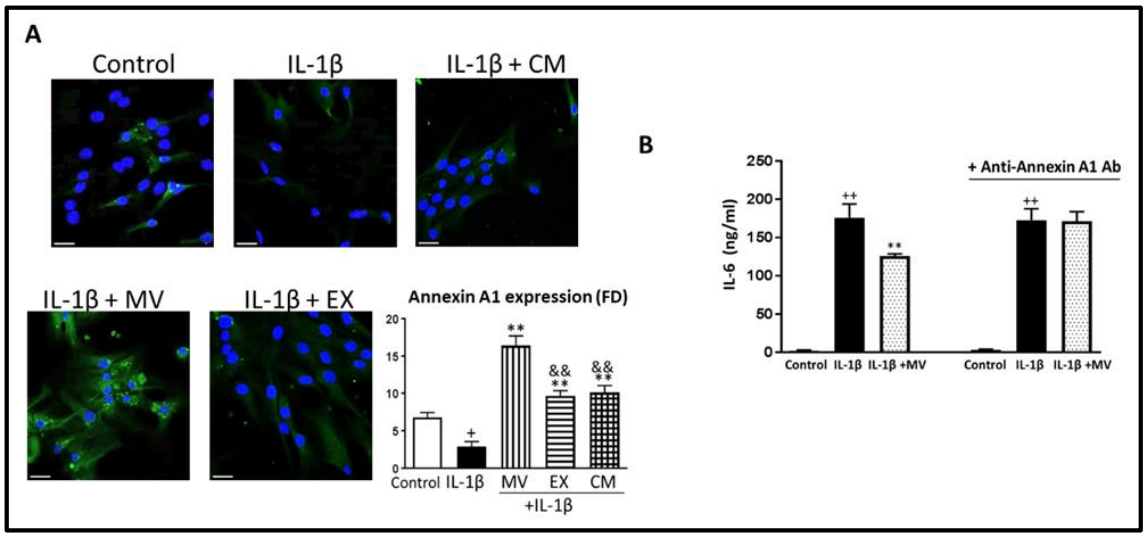

Fig. 9 Annexin A1 expression (A) and IL-6 production after annexin A1 blockade (B) in OA chondrocytes. A: Protein expression was determined by immunofluorescence. Cultures were treated with IL-1β alone or in combination with EV or CM from AD-MSC for 24 h. Results are expressed as mean±SEM from 4 separate experiments with cells from separate donors. FD: fluorescence density. +P<0.05 compared to control (non-stimulated cells); **P<0.01 compared to IL-1β. &&P<0.01 compared to MV. Bar= 30 µm. B: IL-6 was measured by ELISA in cell culture supernatants. Cultures were treated with IL-1β alone or in combination with MV from AD-MSC after annexin A1 blockade with a specific antibody or without blockade. Results are expressed as mean±SEM from 3 separate experiments with cells from separate donors. ++P<0.01 compared to control (non-stimulated cells); **P<0.01 compared to IL-1β.

Discussion

A wide range of evidence indicates that paracrine effects of MSC are a central mechanism of cell therapy promoting tissue regeneration [29, 30]. In line with this view, we have previously shown that CM from AD-MSC exhibits anti-inflammatory properties in OA

chondrocytes [20]. In the present work, we have characterized the EV present in this CM and assessed their possible contribution to its protective actions.

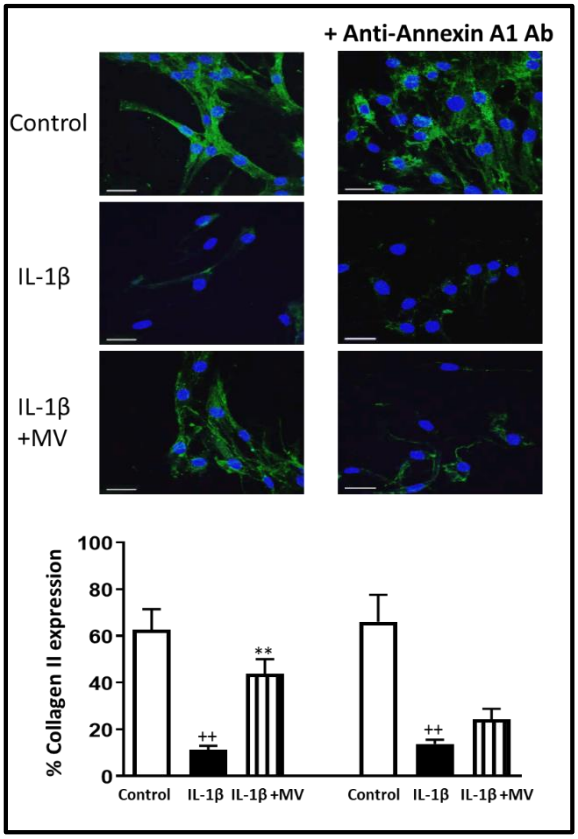

Fig. 10 Collagen II expression after annexin A1 blockade. Collagen II protein expression was determined by immunofluorescence. Cultures were treated with IL-1β alone or in combination with MV from AD-MSC after annexin A1 blockade with a specific antibody or without blockade. Results are expressed as mean±SEM from 3 separate experiments with cells from separate donors. ++P<0.01 compared to control (non-stimulated cells); **P<0.01 compared to IL-1β. Bar= 30 µm.

Synovitis and pro-inflammatory mediators are present in early-stage and late-stage OA (reviewed in [31]) and participate in a positive inflammatory feedback loop in the joint between synoviocytes and chondrocytes likely involving EV [32]. In OA cartilage, IL-1β and TNFα induce a wide range of pro-inflammatory mediators such as cytokines, chemokines, PGE_2, NO and degradative enzymes [31,33]. Therefore, inflammation is associated to progression of cartilage damage in OA and different mediators synergize to amplify and perpetuate the process. Our results have shown that MV and EX present in CM from AD-MSC exert anti-inflammatory effects similar to CM. In contrast, EV from HaCaT cells were completely ineffective on cytokine production in OA chondrocytes and explants confirming the specificity of the observed effects. The downregulation of the pro-inflammatory cytokines TNFα and IL-6 may have implications for the control of altered chondrocyte metabolism. Interestingly, IL-6 has been involved in OA pathophysiology [34] and increased circulating levels of IL-6 have been associated to radiographic knee OA [35].

MV and EX were also able to counteract the inhibitory effects of IL-1β on the anti-inflammatory cytokine IL-10. MV treatment determined a significant IL-10 enhancement compared with EX, which may be related to its content of annexin A1 [36]. The increased production of IL-10 may then contribute to the anti-inflammatory effects of CM and its

EV as this cytokine inhibits the synthesis of pro-inflammatory cytokines [37] and antagonizes their deleterious effects on chondrocyte metabolism [38, 39].

Induction of COX-2 and mPGES-1 and enhanced PGE_2 synthesis in articular chondrocytes lead to anti-anabolic and degradative effects in the joint [25, 40, 41]. Our results indicate that MV and EX are able to control the levels of this eicosanoid through the downregulation of COX-2 and mPGES-1. In addition, MV and EX decreased iNOS induction and NO production, thus preventing the effects of this mediator on the induction and activation of MMPs and the inhibition of extracellular matrix synthesis [42].

The actions of inflammatory mediators in chondrocytes also result in a reduced collagen II expression in OA chondrocytes [27]. Our data indicate that EV from AD-MSC protect OA chondrocytes from the negative effects of IL-1β on collagen II. These findings suggest a role for EV as protective mediators of the differentiated chondrocyte phenotype in inflammatory conditions. Components of extracellular matrix and inflammatory mediators stimulate the degradation of cartilage by inducing different catabolic enzymes. We have shown that MV and to a lesser extent EX, reduced the release of MMP activity. This was accompanied by a significant reduction in gene expression of MMP-13 which plays a key role in collagen II degradation [43]. Taken as a whole, our results suggest that MV may provide better chondroprotection than EX or CM from AD-MSC.

In OA chondrocytes, canonical NF-κB signaling mediates the induction of inflammatory mediators and catabolic mechanisms as well as cellular differentiation changes which favor the onset and perpetuation of disease [44, 45]. A reduction in the activation of this transcription factor by CM and EV could contribute to the observed downregulation of IL-6, TNFα, COX-2, iNOS and MMPs [46]. There is a significant crosstalk of NF-κB with other signaling pathways relevant in OA chondrocytes. In particular, AP-1 cooperates with NF-κB in the induction of MMP-13 and other MMPs [1,47]. Furthermore, IL-1β suppresses collagen II expression in articular chondrocytes by inducing the activation of AP-1 and subsequent suppression of Sox-9 contributing to the loss of the differentiated chondrocyte phenotype [48]. We have shown that MV and EX decrease the DNA binding activities of AP-1 and NF-κB with an effect of MV similar to CM and higher than EX on the last transcription factor. Therefore, the downregulation of MMP activity and MMP-13 gene expression may be the consequence of a decreased activation of both transcription factors.

Proteomic analysis indicated the presence of unique proteins in MV and EX fractions of AD-MSC CM, and some of them can play a role in the regulation of inflammatory processes and immune responses. In particular, annexin A1 is over-represented in MV and exerts complex anti-inflammatory and pro-resolution effects. In addition to the inhibition of different inflammatory mediators, annexin A1 exerts suppressive effect on cells of the immune system (reviewed in [49]). EV represent a way of cellular communication and transfer of components which may be exploited for therapeutic purposes. In this regard, annexin A1 is secreted, at least in part, in EV by different cell types such as neutrophils [50] or human bone marrow mesenchymal stem cells [51] and it can be delivered into the recipient cell [50, 52]. Although further studies are needed to assess the possible contribution of other components of AD-MSC EV, our data suggest that annexin A1 may contribute to the anti-inflammatory and chondroprotective effects of these microparticles under inflammatory stress conditions. These findings are in line with the report that neutrophil MV expressing annexin A1 enhanced chondrocyte anabolic properties *in vitro*, and after *in vivo* administration to mice protected against cartilage degradation in a model of inflammatory arthritis [50].

Conclusion

In summary, we have shown that MV and EX present in the CM of AD-MSC modulate chondrocyte metabolism to counteract the effects of IL-1β. Therefore, EV can reproduce the anti-inflammatory properties of CM from AD-MSC in OA chondrocytes. Our findings are consistent with the hypothesis that EV are mediators of AD-MSC chondroprotective actions with a main role for MV. These EV may play important regulatory roles during cell communication and represent a novel strategy to develop potential treatments in joint conditions.

Acknowledgements

This work was supported by grants SAF2013-4874R (MINECO, FEDER) and PROMETEOII/2014/071 (Generalitat Valenciana), Spain.

Disclosure statement

None.

References

1 Mengshol JA, Vincenti MP, Coon CI, Barchowsky A, Brinckerhoff CE: Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB: differential regulation of collagenase 1 and collagenase 3. Arthritis Rheum 2000;43:801-811.

2 Tetlow LC, Adlam DJ, Woolley DE: Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. Arthritis Rheum 2001;44:585-594.

3 Attur MG, Patel IR, Patel RN, Abramson SB, Amin AR: Autocrine production of IL-1 beta by human osteoarthritis-affected cartilage and differential regulation of endogenous nitric oxide, IL-6, prostaglandin E2, and IL-8. Proc Assoc Am Physicians 1998;110:65-72.

4 Goldring MB, Birkhead J, Sandell LJ, Kimura T, Krane SM: Interleukin 1 suppresses expression of cartilage-specific types II and IX collagens and increases types I and III collagens in human chondrocytes. J Clin Invest 1988;82:2026-2037.

5 Pers YM, Ruiz M, Noël D, Jorgensen C: Mesenchymal stem cells for the management of inflammation in osteoarthritis: state of the art and perspectives. Osteoarthritis Cartilage 2015;23:2027-2035.

6 ter Huurne M, Schelbergen R, Blattes R, Blom A, de Munter W, Grevers LC, Jeanson J, Noël D, Casteilla L, Jorgensen C, van den Berg W, van Lent PLEM: Antiinflammatory and chondroprotective effects of intraarticular injection of adipose-derived stem cells in experimental osteoarthritis. Arthritis Rheum 2012;64:3604-3613.

7 Desando G, Cavallo C, Sartoni F, Martini L, Parrilli A, Veronesi F, Fini M, Giardino R, Facchini A, Grigolo B: Intra-articular delivery of adipose derived stromal cells attenuates osteoarthritis progression in an experimental rabbit model. Arthritis Res Ther 2013;15:R22.

8 Wang Y, Chen X, Cao W, Shi Y: Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. Nat Immunol 2014;15:1009-1016.

9 van Buul GM, Villafuertes E, Bos PK, Waarsing JH, Kops N, Narcisi R, Weinans H, Verhaar JA, Bernsen MR, van Osch GJ: Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explant culture. Osteoarthritis Cartilage 2012;20:1186-1196.

10 Manferdini C, Maumus M, Gabusi E, Piacentini A, Filardo G, Peyrafitte JA, Jorgensen C, Bourin P, Fleury-Cappellesso S, Facchini A, Noel D, Lisignoli G: Adipose-derived mesenchymal stem cells exert antiinflammatory effects on chondrocytes and synoviocytes from osteoarthritis patients through prostaglandin E2. Arthritis Rheum 2013;65:1271-1281.

11 Mause SF, Weber C: Microparticles: protagonists of a novel communication network for intercellular information exchange. Circ Res 2010;107:1047-1057.

12 Colombo M, Raposo G, Théry C: Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol 2014;30:255-289.

13 Lai RC, Chen TS, Lim SK: Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. Regen Med 2011;6:481-492.

14 Maumus M, Jorgensen C, Noel D: Mesenchymal stem cells in regenerative medicine applied to rheumatic diseases: Role of secretome and exosomes. Biochimie 2013;95:2229-2234.

15 Furuta T, Miyaki S, Ishitobi H, Ogura T, Kato Y, Kamei N, Miyado K, Higashi Y, Ochi M: Mesenchymal Stem Cell-Derived Exosomes Promote Fracture Healing in a Mouse Model. Stem Cells Transl Med 2016;5:1620-1630.

16 Sarko DK, McKinney CE: Exosomes: Origins and Therapeutic Potential for Neurodegenerative Disease. Front Neurosci 2017;11:82.

17 Zhang B, Yin Y, Lai RC, Tan SS, Choo AB, Lim SK: Mesenchymal stem cells secrete immunologically active exosomes. Stem Cells Dev 2014;23:1233-1244.

18 Zhang S, Chu WC, Lai RC, Lim SK, Hui JH, Toh WS: Exosomes derived from human embryonic mesenchymal stem cells promote osteochondral regeneration. Osteoarthritis Cartilage 2016;24:2135-2140.

19 Tao SC, Yuan T, Zhang YL, Yin WJ, Guo SC, Zhang CQ: Exosomes derived from miR-140-5p-overexpressing human synovial mesenchymal stem cells enhance cartilage tissue regeneration and prevent osteoarthritis of the knee in a rat model. Theranostics 2017;7:180-195.

20 Platas J, Guillen MI, del Caz MD, Gomar F, Mirabet V, Alcaraz MJ: Conditioned media from adipose tissue-derived mesenchymal stem cells down-regulate degradative mediators induced by interleukin-1â in osteoarthritic chondrocytes. Mediators Inflamm 2013;2013/357014:

21 Platas J, Guillen MI, Perez Del Caz MD, Gomar F, Castejon MA, Mirabet V, Alcaraz MJ: Paracrine effects of human adipose-derived mesenchymal stem cells in inflammatory stress-induced senescence features of osteoarthritic chondrocytes. Aging (Albany NY) 2016;8:1703-1717.

22 Vogel R, Coumans FA, Maltesen RG, Boing AN, Bonnington KE, Broekman ML, Broom MF, Buzas EI, Christiansen G, Hajji N, Kristensen SR, Kuehn MJ, Lund SM, Maas SL, Nieuwland R, Osteikoetxea X, Schnoor R, Scicluna BJ, Shambrook M, de VJ, Mann SI, Hill

AF, Pedersen S: A standardized method to determine the concentration of extracellular vesicles using tunable resistive pulse sensing. J Extracell Vesicles 2016;5:31242.

23 Moroney MA, Alcaraz MJ, Forder RA, Carey F, Hoult JRS: Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids. J Pharm Pharmacol 1988;40:787-792.

24 Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG: A fluorometric assay for the measurement of nitrite in biological samples. Anal Biochem 1993;214:11-16.

25 Li X, Afif H, Cheng S, Martel-Pelletier J, Pelletier JP, Ranger P, Fahmi H: Expression and regulation of microsomal prostaglandin E synthase-1 in human osteoarthritic cartilage and chondrocytes. J Rheumatol 2005;32:887-895.

26 Koshy PJ, Lundy CJ, Rowan AD, Porter S, Edwards DR, Hogan A, Clark IM, Cawston TE: The modulation of matrix metalloproteinase and ADAM gene expression in human chondrocytes by interleukin-1 and oncostatin M: a time-course study using real-time quantitative reverse transcription-polymerase chain reaction. Arthritis Rheum 2002;46:961-967.

27 Goldring MB, Otero M, Tsuchimochi K, Ijiri K, Li Y: Defining the roles of inflammatory and anabolic cytokines in cartilage metabolism. Ann Rheum Dis 2008;67 Suppl 3:iii75-iii82.

28 Perretti M, Dalli J: Exploiting the Annexin A1 pathway for the development of novel anti-inflammatory therapeutics. Br J Pharmacol 2009;158:936-946.

29 Ratajczak MZ, Jadczyk T, Pedziwiatr D, Wojakowski W: New advances in stem cell research: practical implications for regenerative medicine. Pol Arch Med Wewn 2014;124:417-426.

30 Hofer HR, Tuan RS: Secreted trophic factors of mesenchymal stem cells support neurovascular and musculoskeletal therapies. Stem Cell Res Ther 2016;7:131.

31 Goldring MB, Otero M: Inflammation in osteoarthritis. Curr Opin Rheumatol 2011;23:471-478.

32 Withrow J, Murphy C, Liu Y, Hunter M, Fulzele S, Hamrick MW: Extracellular vesicles in the pathogenesis of rheumatoid arthritis and osteoarthritis. Arthritis Res Ther 2016;18:286.

33 Chevalier X: Upregulation of enzymatic activity by interleukin-1 in osteoarthritis. Biomed Pharmacother 1997;51:58-62.

34 Goekoop RJ, Kloppenburg M, Kroon HM, Frolich M, Huizinga TW, Westendorp RG, Gussekloo J: Low innate production of interleukin-1beta and interleukin-6 is associated with the absence of osteoarthritis in old age. Osteoarthritis Cartilage 2010;18:942-947.

35 Livshits G, Zhai G, Hart DJ, Kato BS, Wang H, Williams FM, Spector TD: Interleukin-6 is a significant predictor of radiographic knee osteoarthritis: The Chingford Study. Arthritis Rheum 2009;60:2037-2045.

36 Headland SE, Norling LV: The resolution of inflammation: Principles and challenge. Semin Immunol 2015;27:149-160.

37 Fernandes JC, Martel-Pelletier J, Pelletier JP: The role of cytokines in osteoarthritis pathophysiology. Biorheology 2003;39:237-246.

38 Wang Y, Lou S: Direct protective effect of interleukin-10 on articular chondrocytes in vitro. Chin Med J (Engl) 2001;114:723-725.

39 John T, Muller RD, Oberholzer A, Zreiqat H, Kohl B, Ertel W, Hostmann A, Tschoeke SK, Schulze-Tanzil G: Interleukin-10 modulates pro-apoptotic effects of TNF-alpha in human articular chondrocytes in vitro. Cytokine 2007;40:226-234.

40 Hardy MM, Seibert K, Manning PT, Currie MG, Woerner BM, Edwards D, Koki A, Tripp CS: Cyclooxygenase 2-dependent prostaglandin E2 modulates cartilage proteoglycan degradation in human osteoarthritis explants. Arthritis Rheum 2002;46:1789-1803.

41 Li X, Ellman M, Muddasani P, Wang JH, Cs-Szabo G, Van Wijnen AJ, Im HJ: Prostaglandin E2 and its cognate EP receptors control human adult articular cartilage homeostasis and are linked to the pathophysiology of osteoarthritis. Arthritis Rheum 2009;60:513-523.

42 Lotz M: The role of nitric oxide in articular cartilage damage. Rheum Dis Clin North Am 1999;25:269-282.

43 Goldring MB, Otero M, Plumb DA, Dragomir C, Favero M, El HK, Hashimoto K, Roach HI, Olivotto E, Borzi RM, Marcu KB: Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. Eur Cell Mater 2011;21:202-220.

44 Chockalingam PS, Varadarajan U, Sheldon R, Fortier E, LaVallie ER, Morris EA, Yaworsky PJ, Majumdar MK: Involvement of protein kinase Czeta in interleukin-1beta induction of ADAMTS-4 and type 2 nitric oxide synthase via NF-kappaB signaling in primary human osteoarthritic chondrocytes. Arthritis Rheum 2007;56:4074-4083.

45 Olivotto E, Otero M, Marcu KB, Goldring MB: Pathophysiology of osteoarthritis: canonical NF-kappaB/IKKbeta-dependent and kinase-independent effects of IKKalpha in cartilage degradation and chondrocyte differentiation. RMD Open 2015;1:e000061.

46 Rigoglou S, Papavassiliou AG: The NF-kappaB signaling pathway in osteoarthritis. Int J Biochem Cell Biol 2013;45:2580-2584.

47 Liacini A, Sylvester J, Li WQ, Zafarullah M: Inhibition of interleukin-1-stimulated MAP kinases, activating protein-1 (AP-1) and nuclear factor kappa B (NF-kappa B) transcription factors down-regulates matrix metalloproteinase gene expression in articular chondrocytes. Matrix Biol 2002;21:251-262.

48 Hwang SG, Yu SS, Poo H, Chun JS: c-Jun/activator protein-1 mediates interleukin-1beta-induced dedifferentiation but not cyclooxygenase-2 expression in articular chondrocytes. J Biol Chem 2005;280:29780-29787.

49 D'Acquisto F, Perretti M, Flower RJ: Annexin-A1: a pivotal regulator of the innate and adaptive immune systems. Br J Pharmacol 2008;155:152-169.

50 Headland SE, Jones HR, Norling LV, Kim A, Souza PR, Corsiero E, Gil CD, Nerviani A, Dell'Accio F, Pitzalis C, Oliani SM, Jan LY, Perretti M: Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis. Sci Transl Med 2015;7:315ra190.

51 Kim HS, Choi DY, Yun SJ, Choi SM, Kang JW, Jung JW, Hwang D, Kim KP, Kim DW: Proteomic analysis of microvesicles derived from human mesenchymal stem cells. J Proteome Res 2012;11:839-849.

52 Leoni G, Nusrat A: Annexin A1: shifting the balance towards resolution and repair. Biol Chem 2016;397:971-979.

CAPÍTULO 2

Extracellular vesicles from adipose-derived mesenchymal stem cells downregulate senescence features in osteoarthritic osteoblasts

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Oxidative Medicine and Cellular Longevity (2017) **ID** 7197598.

Extracellular vesicles from adipose-derived mesenchymal stem cells down-regulate senescence features in osteoarthritic osteoblasts

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Abstract

Osteoarthritis (OA) affects all articular tissues leading to pain and disability. The dysregulation of bone metabolism may contribute to the progression of this condition. Adipose-derived mesenchymal stem cells (ASC) are attractive candidates in the search of novel strategies for OA treatment and exert anti-inflammatory and cytoprotective effects on cartilage. Chronic inflammation in OA is a relevant factor in the development of cellular senescence and joint degradation. In this study, we extend our previous observations of ASC paracrine effects to study the influence of conditioned medium and extracellular vesicles from ASC on senescence induced by inflammatory stress in OA osteoblasts. Our results in cells stimulated with interleukin(IL)-1β indicate that conditioned medium, microvesicles and exosomes from ASC down-regulate senescence-associated β-galactosidase activity and the accumulation of γH2AX foci. In addition, they reduced the production of inflammatory mediators, with the highest effect on IL-6 and prostaglandin E_2. The control of mitochondrial membrane alterations and oxidative stress may provide a mechanism for the protective effects of ASC in OA osteoblasts. We have also shown that microvesicles and exosomes mediate the paracrine effects of ASC in these cells. Our study suggests that correction of abnormal osteoblast metabolism by ASC products may contribute to their protective effects in joint degradation.

Keywords: adipose-derived mesenchymal stem cells, osteoblast, senescence, inflammation, oxidative stress, osteoarthritis

Introduction

Osteoarthritis (OA) is the most prevalent joint disease and a leading cause of pain and disability in the aging population. OA affects the whole joint leading to cartilage degradation, synovitis, formation of osteophytes and bone sclerosis. Several studies have demonstrated that bone metabolism is dysregulated in OA and may contribute to the onset and/or progression of this condition [1, 2]. Therefore, the modification of the abnormal metabolism of bone cells may lead to novel approaches for OA treatment [3].

It is known that osteoblasts participate in the regulation of cartilage metabolism and bone remodeling in OA [4]. In particular, subchondral osteoblasts from OA patients show altered phenotypic characteristics [5, 6]. These cells are able to induce a phenotypic shift in OA chondrocytes towards the hypertrophic state [7] as well as the production of matrix metalloproteinases and the inhibition of aggrecan synthesis [8] which play an important role in cartilage degradation [9]. In addition, sites more distal to the joint articular surface show more rigid trabecular bone structure and lower mineralization related to an altered state of trabecular bone remodeling [10].

Pro-inflammatory cytokines are elevated in synovial fluid, synovial membrane, cartilage and subchondral bone and have synergistic effects on inflammation, cartilage degradation and bone remodeling in OA and diseases characterized by bone loss [11-13]. Interleukin(IL)-1β and tumor necrosis factor-α (TNFα) are thought to enhance osteoclastogenesis and bone resorption but they inhibit osteoblast differentiation and bone formation [13, 14]. Additionally, chronic inflammation can lead to cellular senescence in OA [15]. As a model of inflammatory stress, IL-1β stimulation of OA osteoblasts results in metabolic changes and the production of inflammatory and catabolic mediators as well as senescence features [16].

Novel therapeutic approaches for OA are being investigated as there is no pharmacological treatment able to modify the joint structural alterations. Some examples can be the injection of autologous and allogeneic mesenchymal stem cells or the differentiation into cartilage using scaffolds (reviewed in [17]). A wide range of evidence has shown the interest of adipose-derived mesenchymal stem cells (ASC) in tissue regeneration and cytoprotection. For instance, the administration of ASC into the knee joint inhibited synovial activation and prevented cartilage damage in experimental OA

[18, 19]. The cytoprotective and anti-inflammatory properties of ASC in human chondrocytes and experimental OA may be mediated by paracrine effects [20-22] which are also responsible for the inhibition of senescence in OA chondrocytes [23].

There is an increasing interest to know the properties of extracellular vesicles as novel ways of cellular communication [24]. The conditioned medium (CM) of ASC contains extracellular vesicles, mainly microvesicles (MV) and exosomes (EX), which may contribute to the paracrine effects of ASC. In this study, we have extended our previous observations in OA chondrocyte senescence [23] to investigate the contribution of extracellular vesicles to the paracrine effects of ASC on the cellular stress leading to senescence in OA osteoblasts.

Materials and methods

Adipose-derived mesenchymal stem cells

ASC were isolated from the adipose tissue of 8 abdominoplasty-undergone healthy donors (2 men and 6 women, aged 54.4 ± 14.1 years, mean ± SEM). The experimental design was approved by the Institutional Ethical Committees (University of Valencia and La Fe Polytechnic University Hospital, Valencia, Spain). Samples were obtained from donors after they provided informed consent according to the Helsinki Declaration of 1975, as revised in 2013.

Samples were washed with phosphate-buffered saline (PBS), minced and digested at 37ºC for 1 h with 2% of type I collagenase (Gibco, Life Technologies, Madrid, Spain). Tissue remains were filtered through a 100 μm cell strainer (BD Biosciences Durham, NC, USA). Cells were then washed with DMEM/HAM F12 (Sigma-Aldrich, St. Louis, MO, USA) containing penicillin and streptomycin (1%), seeded onto tissue culture flasks (1–2×10^6 cells/mL, 30 ml culture) in DMEM/HAM F12 medium with penicillin and streptomycin (1%), supplemented with 15% extracellular vesicles-free human serum, and incubated at 37º C in a 5% CO_2 atmosphere. Human serum was obtained from whole-blood donations of AB-blood-group-typed donors according to the criteria of Valencia Transfusion Centre. To eliminate the extracellular vesicles fraction, serum was centrifuged during 18 h at 120,000×g and 4ºC using a SW-28 swinging-bucket rotor (Beckman Coulter, CA, USA). When cells reached semi confluence, culture plates were

washed and the ASC phenotype confirmed by flow cytometry (Flow Cytometer II, BD Biosciences, San Jose, CA, USA) using specific antibodies: anti-CD105-PE, antiCD90PerCP-eFluo 710, anti-CD34APC (eBioscience, Inc., San Diego, CA, USA), and anti-CD45-PE (BD Pharmingen) and measuring cellular viability with propidium iodide. Finally, conditioned medium (CM) was collected from ASC culture cells at passage 0 every 48 h of culture. It was pooled, centrifuged, and stored in sterile conditions at −80ºC prior to further use.

Isolation of extracellular vesicles

Vesicles were obtained from the CM of ASC using a filtration/centrifugation-based protocol. Cellular debris was eliminated by centrifugation at 300×g for 10 min. Vesicles were then collected from the supernatant through differential centrifugation steps. CM was filtered through 800 nm filter (Merck, Darmstadt, Germany) and centrifuged at 12,200×g for 20 min at 4°C to pellet microvesicles. Then, supernatants were filtered through 200 nm filter (Merck, Darmstadt, Germany) and centrifuged at 100,000×g for 90 min at 4°C. Pellets were washed once with sterile PBS, resuspended in 15 µL of PBS and stored at -80ºC until further use.

Tunable Resistive Pulse Sensing

Extracellular vesicles preparations were analyzed by Tunable Resistive Pulse Sensing (TRPS) using a qNano instrument (IZON Sciences Ltd., Oxford, UK) as previously described [25]. Briefly, NP100, and NP300 nanopore membranes were used to measure the samples of EX and MV, respectively. At least 500 events/sample were counted. Calibration was performed using calibration beads SKP200 and SKP400, provided by the manufacturer (IZON Sciences Ltd.).

Transmission Electron Microscopy

Preparation of samples for Transmission Electron Microscopy (TEM) was performed by the Microscopy Service (SCSIE, University of Valencia). LR-white resin inclusion was performed. Samples were filtered in resin and polymerized at 60ºC for 48 h. Ultrathin slices (60 nm) were made with a diamond blade (DIATOME, Hartfield, USA) in eyelet grilles in a UC6 Ultracut (Leica, Wetzlar, Germany) and stained with uranyl acetate 2% for 25 min and lead citrate 3% for another 12 min prior to visualization in Jeol-1010

(JEOL Ltd. Tokyo, Japan) at 60 kV. Images were acquired with a digital camera MegaView III with Olympus Image Analysis Software (Olympus, Tokyo, Japan).

OA osteoblasts

Knee specimens were obtained from patients with advanced OA diagnosed (21 women and 9 men, aged 68.4±9.6 years, mean ± SEM) undergoing total knee joint replacement. Diagnosis was based on clinical and radiological evaluation. The experimental design was approved by the Institutional Ethical Committees (University of Valencia and La Fe Polytechnic University Hospital, Valencia, Spain). Samples were obtained from donors after they provided informed consent according to the Helsinki Declaration of 1975, as revised in 2013.

Trabecular bone samples were obtained from the femoral condyles and tibial plateaus, cut into small pieces, and subjected to enzymatic digestion with 1 mg/mL of collagenase type IA (Sigma-Aldrich) at 37°C in DMEM/HAM F-12 (Sigma-Aldrich), containing penicillin and streptomycin (1%) for 2 h. The digested tissue was cultured in osteoblast medium (Promocell, Labclinics S.A., Barcelona, Spain) in a humidified incubator with 5% CO_2 at 37°C. This medium was replaced twice a week. When cells were at 70% of confluence, bone fragments were removed and cells were allowed to grow until confluent. Cell phenotype was characterized by flow cytometry analysis using a Becton Dickinson FACSCanto II cytometer (BD, Franklin Lakes, NJ) and specific antibodies as previously reported [26]. For cell stimulation and treatment, subconfluent osteoblasts were incubated for 24 h in DMEM/HAM F12 (Sigma-Aldrich) containing penicillin and streptomycin (1%) supplemented with 15% extracellular vesicles-free human serum, and stimulated with IL-1β (10 ng/ml) in the presence or absence of MV ($3.6x10^7$ particles/mL), EX ($7.2x10^7$ particles/mL) or CM for 24 h (or 7 days for senescence-associated β-galactosidase activity (SA-β-Gal) experiments). These concentrations are in the range of those present in CM used in the same experiments.

Senescence-associated β-Galactosidase Activity

Osteoblasts were seeded at $20×10^3$ cells/well in Lab-tek chambers (Thermo Scientific, Rochester, NY, USA), then stimulated with IL-1β (10 ng/mL) and treated with MV ($3.6x10^7$ particles/mL) or EX ($7.2x10^7$ particles/mL) or CM (0.2 mL) for 7 days. SA-β-gal activity was measured using the cellular senescence assay kit from Cell Biolabs (San Diego, CA) in its fluorometric format. Briefly, cells were washed with cold PBS and lysed

during 5 minutes at 4ºC. Lysates were centrifuged and supernatant was collected as cell lysate. After transfer to fluorescence 96-well plates, lysates were incubated in presence of assay buffer during 1 h at 37ºC. Reaction was stopped and fluorescence was measured at 360 nm (excitation)/465 nm (emission) in a Victor3 microplate reader (PerkinElmer España, Madrid, Spain).

Immunofluorescence assay for γH2AX foci

Osteoblasts were seeded at 20×10^3 cells/well in Lab-tek chambers (Thermo Scientific, Rochester, NY, USA), then stimulated with IL-1β (10 ng/mL) and treated with MV ($3.6x10^7$ particles/mL) or EX ($7.2x10^7$ particles/mL) or CM (0.2 mL) for 24 h. All cells were fixed with 4% formaldehyde in PBS for 30 min at 4°C, blocked with 5% normal goat serum and 0.3% Triton X-100 in PBS for 60 min at room temperature. Osteoblasts were further incubated with phospho–histone H2AX (Ser139) antibody (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. Finally, cells were incubated with FITC-conjugated goat anti-rabbit IgG (R&D Biosystems, Abingdon, UK), mounted in Prolong Gold antifade reagent with DAPI, and examined under a confocal microscope (Olympus FV1000, Tokyo, Japan).

Enzyme-Linked Immunosorbent Assay

Osteoblasts were stimulated with IL-1β (10 ng/mL) in presence or absence of MV ($3.6x10^7$ particles/mL), EX ($7.2x10^7$ particles/mL) or CM (1 mL) for 24 h. Supernatants were harvested, centrifuged, and frozen at -80º C until analysis. In order to measure the levels of 4-hydroxy-nonenal (HNE)-proteins, cells were lysed with 1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl and 25 mM Tris, pH 7.4 buffer. Lysates were centrifuged at 4ºC for 10 min at 10,000×g. Then, 4-HNE-modified proteins were measured with the Cell Biolabs ELISA kit (San Diego, CA, USA) with sensitivity of 1.56 µg/mL. TNFα, IL-6, and IL-10 were measured in supernatants with ELISA kits from eBioscience (San Diego, CA, USA) with a sensitivity of 4.0 pg/mL for TNFα and IL-6, and 2.0 pg/mL for IL-10.

Determination of prostaglandin E_2

Osteoblasts were stimulated with IL-1β (10 ng/mL) in presence or absence of MV ($3.6x10^7$ particles/mL), EX ($7.2x10^7$ particles/mL) or CM (1 mL) for 24 h. Supernatants

were used to measure prostaglandin E_2 (PGE_2) by radioimmunoassay as previously described [27] using a Victor3 microplate reader (PerkinElmer España, Madrid, Spain).

Mitochondrial Membrane Potential

Osteoblasts were stimulated with IL-1β (10 ng/mL) in presence or absence of MV ($3.6x10^7$ particles/mL), EX ($7.2x10^7$ particles/mL) or CM (1 mL) for 24 h. Then, mitochondrial transmembrane potential (Δψm) was assessed with the JC-1 probe (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolylcarbocyanine iodide, Thermo Scientific, Rochester, NY, USA). This lipophilic membrane-permeant cation exhibit potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from ~525 nm (monomeric form) to~590 nm (aggregated form). Cell cultures were trypsinized, resuspended in 1 mL of PBS, and incubated with 10 μg/mL of JC-1 dye for 15 min at 37ºC and 5% CO_2. Both red and green fluorescence emissions were analyzed by flow cytometry using an excitation wavelength of 488 nm and observation wavelengths of 530 nm for green fluorescence and 585 nm for red fluorescence, and a Becton Dickinson FACSCanto II cytometer (BD, Franklin Lakes, NJ, USA).

Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by Sidak's post-test using the GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA). A *P* value of less than 0.05 was considered to be significant.

Results

Characterization of MV and EX from ASC

MV and EX fractions were isolated as indicated in Materials and methods. TRPS analysis indicated a mean diameter of 316 nm and 115 nm, and a concentration of $8x10^9$ and $3.8x10^{10}$ particles/mL for MV and EX, respectively. Figure 1 shows a representative TRPS analysis of MV (A) and EX (B) fractions. The morphology of MV and EX was studied by TEM (data not shown).

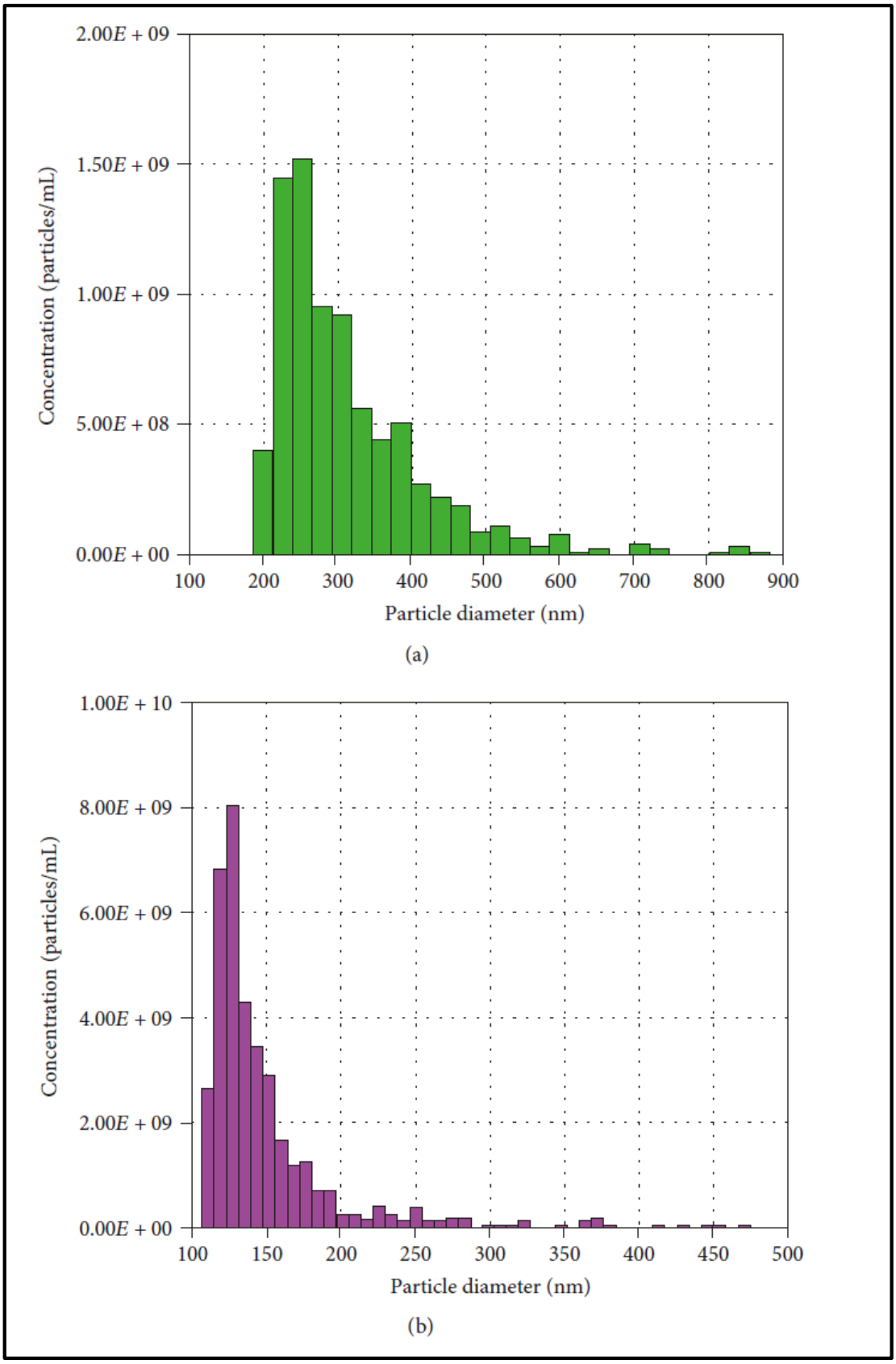


Fig. 1 Characterization of MV and EX isolated from CM. Representative TRPS analysis of MV (A) and EX (B).

SA-β-gal activity induced by IL-1β in human OA osteoblasts

We examined SA-β-gal activity in OA osteoblasts for the effects of CM and extracellular vesicles on this marker of cellular senescence. Figure 2 shows that IL-1β stimulation for 7 days enhanced SA-β-gal activity by 57% with respect to control (non-stimulated cells). We found that treatment with MV, EX or CM resulted in similar effects with a significant reduction of this activity by 48% with respect to IL-1β.

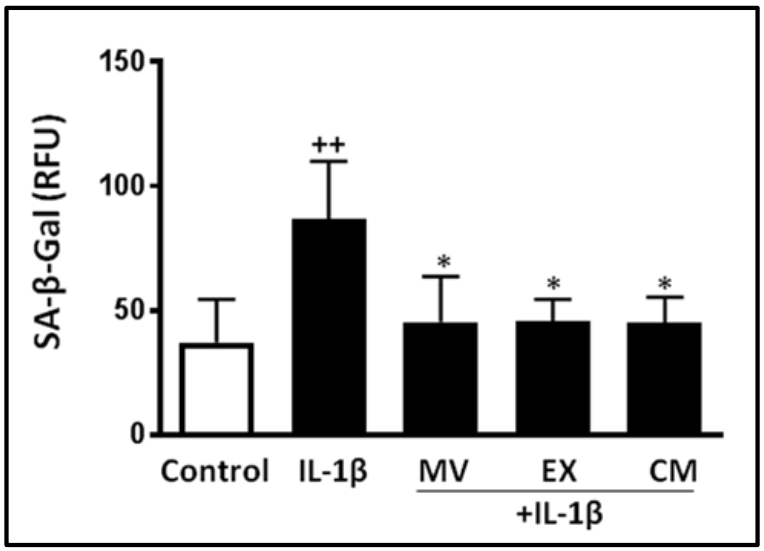
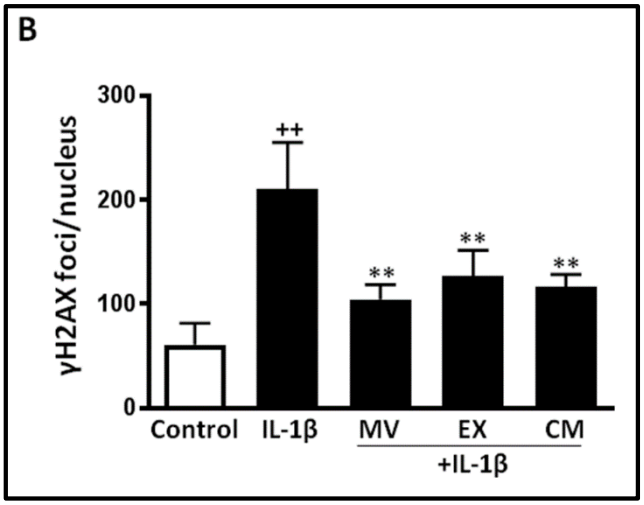


Fig. 2 SA-β-Gal activity in OA osteoblasts. Cultures were treated with IL-1β alone or in combination with MV, EX or CM for 7 days. SA-β-Gal activity was measured by using the cellular senescence assay kit (Cell Biolabs) and expressed as relative fluorescence units (RFU). Results show mean±SD from 4 separate experiments with cells from separate donors. ++P<0.01 compared to control (non-stimulated cells); *P<0.05 compared to IL-1β.

γH2AX foci accumulation

The presence of phosphorylated histone H2AX indicates DNA damage and correlates with age [28]. To assess the effect of CM and extracellular vesicles, γH2AX foci were quantified in nuclei. The immunofluorescence analysis showed that γH2AX foci were increased in the presence of IL-1β for 24 h by 70% compared with control (non-stimulated cells) (Figure 3A and B). The amount of γH2AX foci per nucleus was significantly reduced by treatment with MV (46%), EX (44%) or CM (30%).



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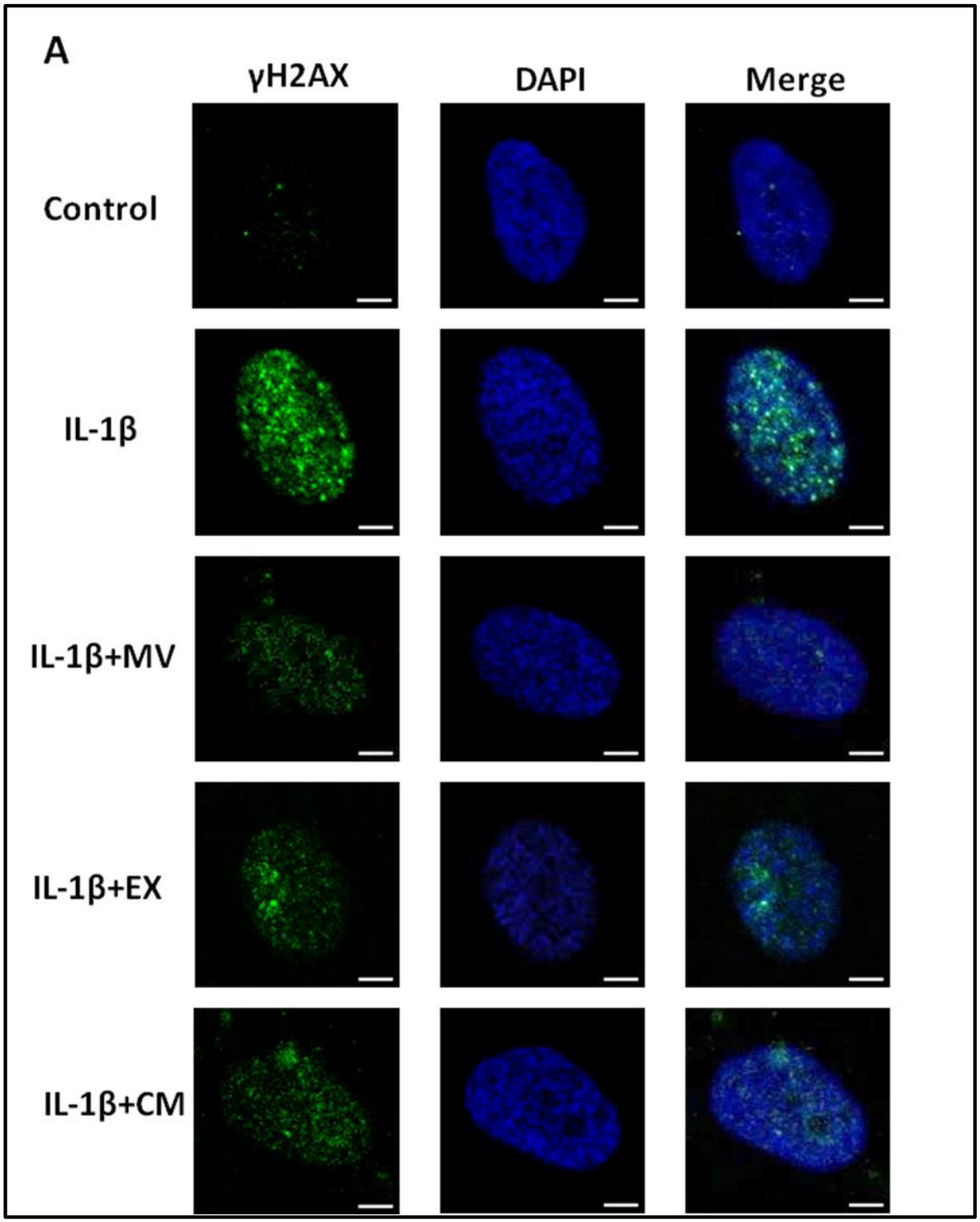


Fig. 3 Immunofluorescence analysis of γH2AX foci accumulation. A: Representative images. γH2AX foci (green, FITC fluorescence), nuclei were stained with DAPI (blue). B: number of γH2AX foci per nucleus. Cultures were treated with IL-1β alone or in combination with MV, EX or CM for 24 h. Bar= 5 µm. Results are expressed as mean±SD from 3 separate experiments with cells from separate donors. ++P<0.01 compared to control (non-stimulated cells); **P<0.01 compared to IL-1β.

Production of pro-inflammatory and anti-inflammatory mediators

Inflammation is involved in cellular senescence and OA. We have determined the production of key pro-inflammatory mediators in OA osteoblasts. After 24 h of incubation, IL-1β strongly induced the production of pro-inflammatory cytokine IL-6 and the eicosanoid PGE_2 while TNFα levels were enhanced to a lower extent (Figure 4). Treatment with MV, EX or CM did not affect the basal release of these mediators. Nevertheless, MV, EX and CM significantly reduced IL-6 and PGE_2, and CM also decreased TNFα levels in cells stimulated with IL-1β. In addition, the anti-inflammatory cytokine IL-10 was measured in this system. As shown in Figure 5, after MV, EX or CM treatment the levels of IL-10 significantly increased by more than three-fold after 24 h of incubation in the presence of IL-1β.

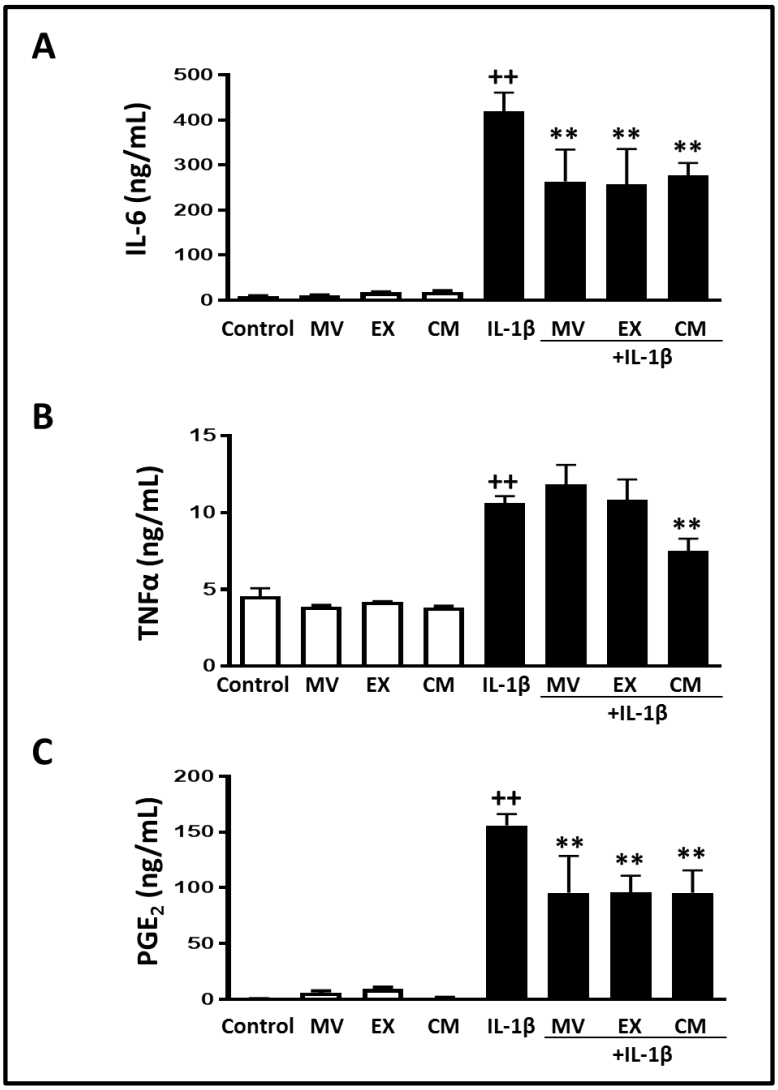


Fig. 4 Release of inflammatory mediators. IL-6 (A) and TNFα (B) were measured by ELISA; PGE2 (C) was measured by radioimmunoassay in cell culture supernatants of OA osteoblasts. Cultures were treated with IL-1β and/or MV, EX or CM for 24 h. Results are expressed as mean±SD from 4 separate experiments with cells from separate donors. ++P<0.01 compared to control (non-stimulated cells); **P<0.01 compared to IL-1β.

Oxidative stress

As oxidative stress is a key process in the induction of cellular senescence [29], we next investigated the effects of CM, MV and EX on protein modification by oxidative stress. As shown in Figure 6, IL-1β induced the production of oxidative stress leading to the accumulation of HNE-modified proteins in OA osteoblasts. We observed a significant

reduction (by 50 %) in the amount of HNE-protein adducts measured in cells treated with CM, MV or EX.

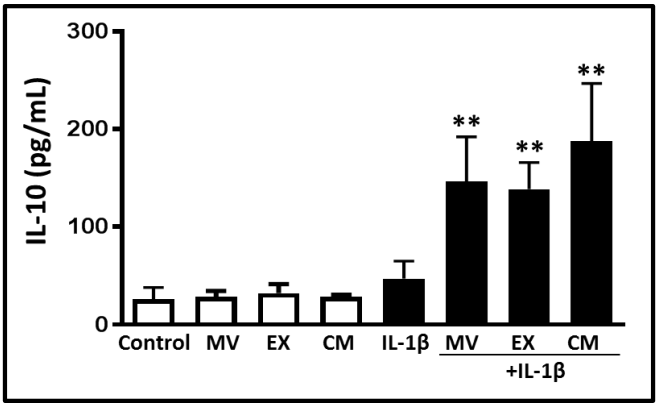


Fig. 5 Release of IL-10 by OA osteoblasts. IL-10 was measured by ELISA in cell culture supernatants. Cultures were treated with IL-1β and/or MV, EX or CM for 24 h. Results are expressed as mean±SD from 5 separate experiments with cells from separate donors. **P<0.01 compared to IL-1β.

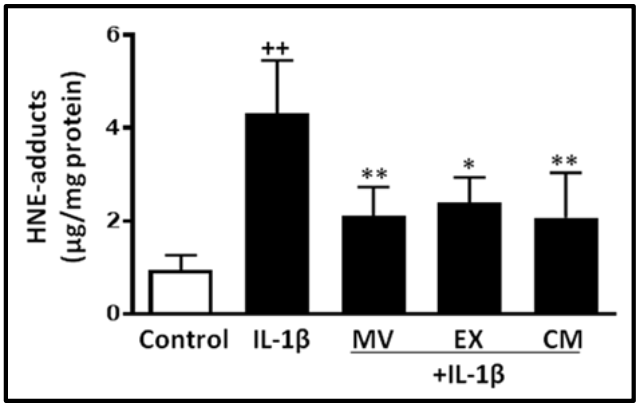
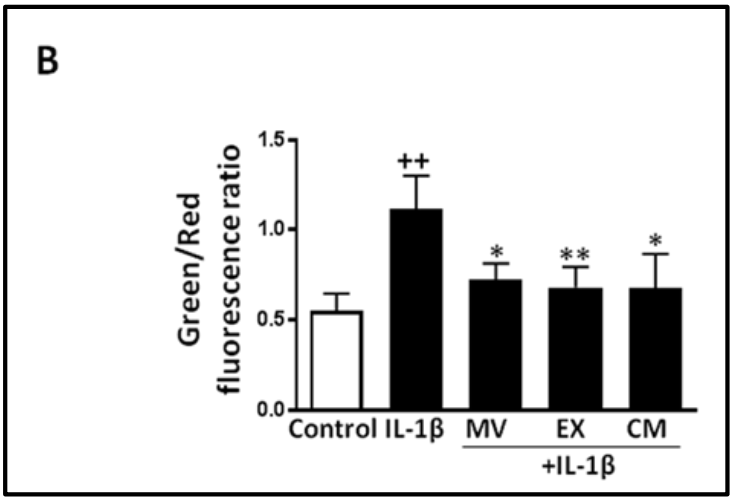


Fig. 6 Quantification of HNE-protein adducts in OA osteoblasts. HNE-protein adducts were measured by ELISA in cellular extracts. Cultures were treated with IL-1β alone or in combination with MV, EX or CM for 24 h. Results are expressed as mean±SD from 4 separate experiments with cells from separate donors. ++P<0.01 compared to control (non-stimulated cells); *P<0.05, **P<0.01 compared to IL-1β.

Mitochondrial membrane potential

To measure changes in the mitochondrial membrane potential (ΔΨ) we have used the probe JC-1. Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. Incubation of OA osteoblasts with IL-1β increased the green/red ratio by two-fold indicating a lowering of the mitochondrial membrane potential (Figure 7A and B). Treatment with CM, MV or EX significantly restored the mitochondrial membrane potential.



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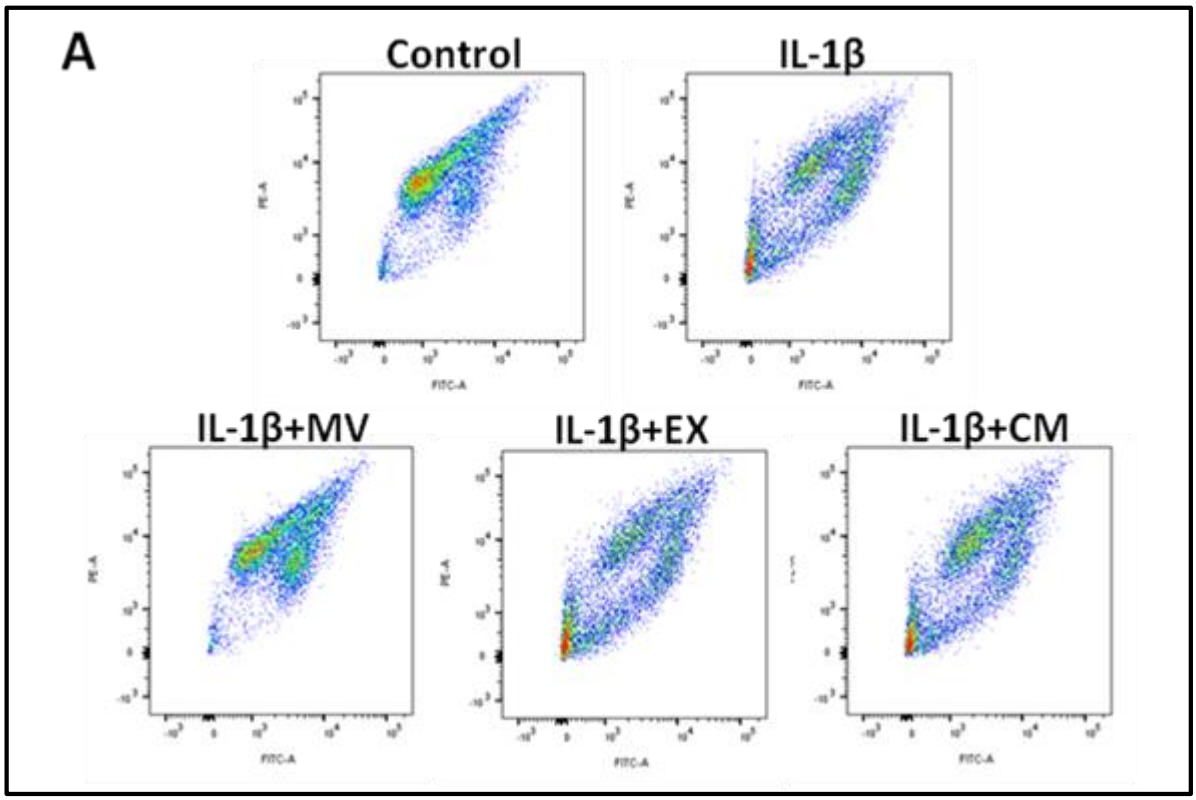

Fig. 7 Analysis of mitochondrial membrane potential in OA osteoblasts. Analysis was performed by flow cytometry using the probe JC-1. Representative images (A); green/red fluorescence ratio (B). Cultures were treated with IL-1β alone or in combination with MV, EX or CM for 24 h. Results are expressed as mean±SD from 3 separate experiments with cells from separate donors. ++P<0.01 compared to control (non-stimulated cells); *P<0.05, **P<0.01 compared to IL-1β.

Discussion

Multiple types of stress can lead to premature cellular senescence. It has been proposed that low-grade chronic inflammation during aging and associated pathologies can lead to oxidative stress and cell alterations driving senescence. Therefore, oxidative stress induces telomere-independent senescence leading to cell dysfunction [30]. Senescent cells develop a senescence-associated secretory phenotype with production of cytokines such as IL-6, growth factors or matrix metalloproteinases which are mediators of complex autocrine and paracrine effects leading to phenotypic changes in nearby cells and alterations of tissue microenvironment [31]. Therefore, the accumulation of senescent cells with aging results in tissue or organ dysfunction. In support of this theory, it has been demonstrated that elimination of senescent cells in mice delays age-related pathologies [32].

There is increasing evidence that chronic inflammation-related senescence and aging may contribute to the development of OA [33]. The majority of studies of cellular senescence in OA have focused on chondrocytes. Chondrocyte senescence has been detected in OA cartilage [34] where the accumulation of cells could contribute to tissue destruction [35, 36]. ASC may offer new therapeutic approaches to regulate premature senescence. Recently, we have reported that ASC and CM inhibit senescence in OA chondrocytes [23]. In the current work, we have demonstrated the paracrine effects of ASC to down-regulate senescence features induced by inflammatory stress in OA osteoblasts as well as the relevant contribution of MV and EX.

Joint tissues release pro-inflammatory cytokines in response to a wide variety of agents leading to mitochondrial changes, increased synthesis of reactive oxygen species (ROS) and DNA alterations which can induce premature senescence. In osteoblasts, cellular senescence is an important mechanism of age-related dysfunction which causes bone loss [37]. Aging bone shows a reduced ability of response against mechanical stress linked to some characteristics such as intralacunar hypermineralization and lower osteocyte lacunar density [38] which are also present in OA [39]. Subchondral bone alterations and cartilage degeneration are important processes during OA progression [40]. Interestingly, transplantation of senescent fibroblasts into the knee joint region of mice induces an inflammatory response and alterations in cartilage and bone resembling OA [41].

Pro-inflammatory and catabolic mediators produced by subchondral bone may contribute to cartilage and bone changes. It is considered that osteoblast cytokines can transit the subchondral bone plate and calcified cartilage and communicate with chondrocytes [42]. Therefore, osteoblasts produce IL-6 which regulates the balance of bone resorption and formation during bone remodeling and can promote matrix degradation directly in both bone and cartilage [43]. We have demonstrated the paracrine anti-inflammatory effects of ASC on OA osteoblasts, with down-regulation of IL-6 and TNFα. In addition, our results indicate that MV and EX could be the mediators of ASC paracrine effects on IL-6 which is the inflammatory marker showing the strongest association with age-related disease and fragility [33]. In contrast, MV and EX did not significantly reduced the levels of TNFα suggesting that soluble mediators present in CM may be the factors responsible for the regulation of this cytokine. The high levels of PGE_2 produced in our model of inflammatory stress were also reduced by CM and extracellular vesicles. The production of this eicosanoid is enhanced during cellular senescence in human fibroblasts [44].

Concerning bone metabolism, PGE_2 stimulates bone formation at low concentrations but it may be inhibitory at high concentrations [45, 46] and this eicosanoid may be a mediator of osteoclastogenesis induced by IL-6 [47]. In addition, PGE_2 may be an enhancing factor for IL-6 production in human osteoblasts [48]. Therefore, our results suggest that a decrease in PGE_2 production contributes to the anti-inflammatory and anti-senescence effects of ASC and it may help to counteract the consequences of chronic inflammation on bone metabolism. In addition, we have shown that CM, MV and EX from ASC enhance the production of the anti-inflammatory cytokine IL-10 in the presence of IL-1β which may prolong the down-regulation of the inflammatory response as this cytokine inhibits the production of ROS and pro-inflammatory cytokines by macrophages [49, 50], and PGE_2 by OA synovial fibroblasts [51]. This effect of CM, MV and EX on IL-10 is in line with that reported for CM in OA chondrocytes [52]. Of note, IL-10 has been proposed as a treatment option for inflammation-related bone loss [53].

Chronic oxidative stress related to ageing or mechanical stress may lead to cellular senescence in joint tissues [54] and age-related alterations in osteoblast differentiation and function [37, 55]. The majority of ROS are produced by the mitochondria as a consequence of oxidative phosphorylation which generates a potential energy for protons ($\Delta\Psi$) across the mitochondrial inner membrane. ROS generated within the mitochondria can damage mitochondrial components and nuclear DNA, besides inducing the oxidative modification of proteins and the activation of different signaling pathways [56]. We have examined whether the control of oxidative stress could be involved in the protective effects of CM and extracellular vesicles observed in OA osteoblasts. The results of our analysis indicate that CM, MV and EX from ASC significantly down-regulate the mitochondrial membrane changes and oxidative stress induced by IL-1β, thus providing a plausible mechanism to inhibit cellular senescence. Mitochondrial ROS are linked to senescence through nuclear DNA damage [57]. The phosphorylation of H2AX following DNA double-strand breaks increases with age and may be a biomarker for human morbidity in age-related diseases [28]. We found that CM and extracellular vesicles from ASC are able to reduce DNA damage as shown by a lower accumulation of γH2AX foci which may be a consequence of oxidative stress control.

As osteoblasts play an important role in the regulation of cartilage metabolism and bone remodeling the correction of the abnormal cell metabolism may offer novel therapeutic approaches for joint degradation. Further research into the mechanisms by which

senescence of different articular cells contribute to OA is needed to uncover novel targets useful to prevent or treat this condition.

In conclusion, we have shown that CM and extracellular vesicles from ASC down-regulate inflammation and oxidative stress which may mediate anti-senescence effects in OA osteoblasts. Our data also indicate that MV and EX from ASC are responsible for the paracrine effects of these cells and suggest the interest of these extracellular vesicles to develop new treatments for joint conditions.

Acknowledgements

This work has been funded by grants SAF2013-48724R (MINECO, FEDER) and PROMETEOII/2014/071 (Generalitat Valenciana).

Conflicts of Interest

The authors have no conflicts of interest to disclose.

References

[1] J.P. Mansell and A.J. Bailey, "Abnormal cancellous bone collagen metabolism in osteoarthritis", *The Journal of Clinical Investigation*, vol.101, no.8, pp.1596-1603, 1998.

[2] Y. Henrotin, L. Pesesse, and C. Sanchez, "Subchondral bone and osteoarthritis: biological and cellular aspects", *Osteoporosis International*, vol.23 Suppl 8, pp.S847-S851, 2012.

[3] C. Boileau, S.K. Tat, J.P. Pelletier, S. Cheng, and J. Martel-Pelletier, "Diacerein inhibits the synthesis of resorptive enzymes and reduces osteoclastic differentiation/survival in osteoarthritic subchondral bone: a possible mechanism for a protective effect against subchondral bone remodelling", *Arthritis Research and Therapy*, vol.10, no.3, pp.R71, 2008.

[4] C. Westacott, "Interactions between subchondral bone and cartilage in OA. Cells from osteoarthritic bone can alter cartilage metabolism", *Journal of Musculoskeletal Neuronal Interactions*, vol.2, no.6, pp.507-509, 2002.

[5] G. Hilal, J. Martel-Pelletier, J.P .Pelletier, P. Ranger, and D. Lajeunesse, "Osteoblast-like cells from human subchondral osteoarthritic bone demonstrate an altered phenotype in vitro: possible role in subchondral bone sclerosis", *Arthritis and Rheumatism*, vol.41, no.5, pp.891-899, 1998.

[6] D. Couchourel, I. Aubry, A. Delalandre, et al., "Altered mineralization of human osteoarthritic osteoblasts is attributable to abnormal type I collagen production", *Arthritis and Rheumatism*, vol.60, no.5, pp.1438-1450, 2009.

[7] C. Sanchez, M.A. Deberg, N. Piccardi, P. Msika, J.Y. Reginster, and Y.E. Henrotin, "Subchondral bone osteoblasts induce phenotypic changes in human osteoarthritic chondrocytes", *Osteoarthritis and Cartilage*, vol.13, no.11, pp.988-997, 2005.

[8] C. Sanchez, M.A. Deberg, N. Piccardi, P. Msika, J.Y. Reginster, and Y.E. Henrotin, "Osteoblasts from the sclerotic subchondral bone downregulate aggrecan but upregulate metalloproteinases expression by chondrocytes. This effect is mimicked by interleukin-6, -1beta and oncostatin M pre-treated non-sclerotic osteoblasts", *Osteoarthritis and Cartilage*, vol.13, no.11, pp.979-987, 2005.

[9] C.I. Westacott, G.R. Webb, M.G. Warnock, J.V. Sims, and C.J. Elson, "Alteration of cartilage metabolism by cells from osteoarthritic bone", *Arthritis and Rheumatism*, vol.40, no.7, pp.1282-1291, 1997.

[10] D.D. Kumarasinghe, E. Perilli, H. Tsangari, et al., "Critical molecular regulators, histomorphometric indices and their correlations in the trabecular bone in primary hip osteoarthritis", *Osteoarthritis and Cartilage*, vol.18, no.10, pp.1337-1344, 2010.

[11] H. Hulejova, V. Baresova, Z. Klezl, M. Polanska, M. Adam, and L. Senolt, "Increased level of cytokines and matrix metalloproteinases in osteoarthritic subchondral bone", *Cytokine*, vol.38, no.3, pp.151-156, 2007.

[12] P. Wojdasiewicz, L.A. Poniatowski, and D. Szukiewicz, "The role of inflammatory and anti-Inflammatory cytokines in the pathogenesis of osteoarthritis", *Mediators of Inflammation*, vol.2014, pp.561459, 2014.

[13] P. Lencel and D. Magne, "Inflammaging: the driving force in osteoporosis?", *Medical Hypotheses*, vol.76, no.3, pp.317-321, 2011.

[14] D.C. Lacey, P.J. Simmons, S.E. Graves, and J.A. Hamilton, "Proinflammatory cytokines inhibit osteogenic differentiation from stem cells: implications for bone repair during inflammation", *Osteoarthritis and Cartilage*, vol.17, no.6, pp.735-742, 2009.

[15] R.F. Loeser, "Aging processes and the development of osteoarthritis", *Current Opinion in Rheumatology*, vol.25, no.1, pp.108-113, 2013.

[16] V. Clerigues, M.I. Guillen, M.A. Castejon, F. Gomar, V. Mirabet, and M.J. Alcaraz, "Heme oxygenase-1 mediates protective effects on inflammatory, catabolic and senescence responses induced by interleukin-1beta in osteoarthritic osteoblasts", *Biochemical Pharmacology*, vol.83, pp.395-405, 2012.

[17] Y.M. Pers, M. Ruiz, D. Noël, and C. Jorgensen, "Mesenchymal stem cells for the management of inflammation in osteoarthritis: state of the art and perspectives", *Osteoarthritis and Cartilage*, vol.23, no.11, pp.2027-2035, 2015.

[18] M. ter Huurne, R. Schelbergen, R. Blattes, et al., "Antiinflammatory and chondroprotective effects of intraarticular injection of adipose-derived stem cells in experimental osteoarthritis", *Arthritis and Rheumatism*, vol.64, no.11, pp.3604-3613, 2012.

[19] G. Desando, C. Cavallo, F. Sartoni, et al., "Intra-articular delivery of adipose derived stromal cells attenuates osteoarthritis progression in an experimental rabbit model", *Arthritis Research and Therapy*, vol.15, no.1, pp.R22, 2013.

[20] C. Manferdini, M. Maumus, E. Gabusi, et al., "Adipose-derived mesenchymal stem cells exert antiinflammatory effects on chondrocytes and synoviocytes from osteoarthritis patients through prostaglandin E2", *Arthritis and Rheumatism*, vol.65, no.5, pp.1271-1281, 2013.

[21] M. Maumus, C. Manferdini, K. Toupet, et al., "Adipose mesenchymal stem cells protect chondrocytes from degeneration associated with osteoarthritis", *Stem Cell Research*, vol.11, no.2, pp.834-844, 2013.

[22] K. Kuroda, T. Kabata, K. Hayashi, et al., "The paracrine effect of adipose-derived stem cells inhibits osteoarthritis progression", *BMC Musculoskeletal Disorders*, vol.16, pp.236, 2015.

[23] J. Platas, M.I. Guillen, M.D. Perez Del Caz, et al., "Paracrine effects of human adipose-derived mesenchymal stem cells in inflammatory stress-induced senescence features of osteoarthritic chondrocytes", *Aging (Albany NY)*, vol.8, no.8, pp.1703-1717, 2016.

[24] M. Colombo, G. Raposo, and C. Théry, "Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles", *Annual Review of Cell and Developmental Biology*, vol.30, pp.255-289, 2014.

[25] R. Vogel, F.A. Coumans, R.G. Maltesen, et al., "A standardized method to determine the concentration of extracellular vesicles using tunable resistive pulse sensing", *Journal of Extracellular Vesicles*, vol.5, pp.31242, 2016.

[26] J. Platas, M.I. Guillen, F. Gomar, M.A. Castejon, P. Esbrit, and M.J. Alcaraz, "Anti-senescence and Anti-inflammatory Effects of the C-terminal Moiety of PTHrP Peptides in OA Osteoblasts", *Journal of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 72, no.5, pp. 624-631, 2017.

[27] M.A. Moroney, M.J. Alcaraz, R.A. Forder, F. Carey, and J.R.S. Hoult, "Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids", *Journal of Pharmacy and Pharmacology*, vol.40, no.11, pp.787-792, 1988.

[28] S.H. Schurman, C.A. Dunn, R. Greaves, et al., "Age-related disease association of endogenous gamma-H2AX foci in mononuclear cells derived from leukapheresis", *PLoS One*, vol.7, no.9, pp.e45728, 2012.

[29] I. Ben Porath and R.A. Weinberg, "The signals and pathways activating cellular senescence", *International Journal of Biochemistry and Cell Biology*, vol.37, no.5, pp.961-976, 2005.

[30] K. Itahana, J. Campisi, and G.P. Dimri, "Mechanisms of cellular senescence in human and mouse cells", *Biogerontology*, vol.5, no.1, pp.1-10, 2004.

[31] T. Tchkonia, Y Zhu, D.J. Van, J. Campisi, and J.L. Kirkland, "Cellular senescence and the senescent secretory phenotype: therapeutic opportunities", *The Journal of Clinical Investigation*, vol.123, no.3, pp.966-972, 2013.

[32] D.J. Baker, T. Wijshake, T. Tchkonia, et al., "Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders", *Nature*, vol.479, no.7372, pp.232-236, 2011.

[33] M.A. Greene and R.F. Loeser, "Aging-related inflammation in osteoarthritis", *Osteoarthritis and Cartilage*, vol.23, no.11, pp.1966-1971, 2015.

[34] J.S. Price, J.G. Waters, C. Darrah, et al., "The role of chondrocyte senescence in osteoarthritis", *Aging Cell*, vol.1, no.1, pp.57-65, 2002.

[35] R.F. Loeser, "Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix", *Osteoarthritis and Cartilage*, vol.17, no.8, pp.971-979, 2009.

[36] D. Philipot, D. Guerit, D. Platano, et al., "p16INK4a and its regulator miR-24 link senescence and chondrocyte terminal differentiation-associated matrix remodelling in osteoarthritis", *Arthritis Research and Therapy*, vol.16, no.1, pp.R58, 2014.

[37] M. Kassem and P.J. Marie, "Senescence-associated intrinsic mechanisms of osteoblast dysfunctions", *Aging Cell*, vol.10, no.2, pp.191-197, 2011.

[38] B. Busse, D. Djonic, P. Milovanovic, et al., "Decrease in the osteocyte lacunar density accompanied by hypermineralized lacunar occlusion reveals failure and delay of remodeling in aged human bone", *Aging Cell*, vol.9, no.6, pp.1065-1075, 2010.

[39] V.T. Carpentier, J. Wong, Y.Yeap, et al., "Increased proportion of hypermineralized osteocyte lacunae in osteoporotic and osteoarthritic human trabecular bone: implications for bone remodeling", *Bone*, vol.50, no.3, pp.688-694, 2012.

[40] C.I. Westacott, A.F. Barakat, L. Wood, et al., "Tumor necrosis factor alpha can contribute to focal loss of cartilage in osteoarthritis", *Osteoarthritis and Cartilage*, vol.8, no.3, pp.213-221, 2000.

[41] M. Xu, E.W. Bradley, M.M. Weivoda, et al., "Transplanted senescent cells induce an osteoarthritis-like condition in mice", *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 72, no.6, pp. 780-785, 2017.

[42] D.M. Findlay and G.J. Atkins, "Osteoblast-chondrocyte interactions in osteoarthritis", *Current osteoporosis reports*, vol.12, no.1, pp.127-134, 2014.

[43] K. Sakao, K.A. Takahashi, O. Mazda, et al., "Enhanced expression of interleukin-6, matrix metalloproteinase-13, and receptor activator of NF-kappaB ligand in cells derived from osteoarthritic subchondral bone", *Journal of Orthopedic Science*, vol.13, no.3, pp.202-210, 2008.

[44] S. Martien, O. Pluquet, C. Vercamer, et al., "Cellular senescence involves an intracrine prostaglandin E2 pathway in human fibroblasts", *Biochimica et Biophysica Acta*, vol.1831, no.7, pp.1217-1227, 2013.

[45] L.G. Raisz, "Prostaglandins and bone: physiology and pathophysiology", *Osteoarthritis and Cartilage*, vol.7, no.4, pp.419-421, 1999.

[46] K.A. Blackwell, L.G. Raisz, and C.C. Pilbeam, "Prostaglandins in bone: bad cop, good cop?", *Trends in Endocrinology and Metabolism*, vol.21, no.5, pp.294-301, 2010.

[47] H. Tai, C. Miyaura, C.C. Pilbeam, et al., "Transcriptional induction of cyclooxygenase-2 in osteoblasts is involved in interleukin-6-induced osteoclast formation", *Endocrinology*, vol.138, no.6, pp.2372-2379, 1997.

[48] F. Massicotte, D. Lajeunesse, M. Benderdour, et al., "Can altered production of interleukin-1beta, interleukin-6, transforming growth factor-beta and prostaglandin E(2) by isolated human subchondral osteoblasts identify two subgroups of osteoarthritic patients", *Osteoarthritis and Cartilage*, vol.10, no.6, pp.491-500, 2002.

[49] S. Dokka, X. Shi, S. Leonard, L. Wang, V. Castranova, and Y. Rojanasakul, "Interleukin-10-mediated inhibition of free radical generation in macrophages", *American Journal of Physiology - Lung Cellular and Molecular Physiology*, vol.280, no.6, pp.L1196-L1202, 2001.

[50] D.F. Fiorentino, A. Zlotnik, T.R. Mosmann, M. Howard, and A. O'Garra, "IL-10 inhibits cytokine production by activated macrophages", *Journal of Immunology*, vol.147, no.11, pp.3815-3822, 1991.

[51] N. Alaaeddine, J.A. Di Battista, J.P. Pelletier, K. Kiansa, J.M. Cloutier, and J. Martel-Pelletier, "Inhibition of tumor necrosis factor alpha-induced prostaglandin E2 production by the antiinflammatory cytokines interleukin-4, interleukin-10, and interleukin-13 in osteoarthritic synovial fibroblasts: distinct targeting in the signaling pathways", *Arthritis and Rheumatism*, vol.42, no.4, pp.710-718, 1999.

[52] J. Platas, M.I. Guillen, M.D. del Caz, F. Gomar, V. Mirabet, and M.J. Alcaraz, "Conditioned media from adipose tissue-derived mesenchymal stem cells down-regulate degradative mediators induced by interleukin-1beta in osteoarthritic chondrocytes", *Mediators of Inflammation*, vol.2013/357014, 2013.

[53] Q. Zhang, B. Chen, F. Yan, et al., "Interleukin-10 inhibits bone resorption: a potential therapeutic strategy in periodontitis and other bone loss diseases", *BioMed Research International*, vol.2014, pp.284836, 2014.

[54] K. McCulloch, G.J. Litherland, and T.S. Rai, "Cellular senescence in osteoarthritis pathology", *Aging Cell*, vol.16, no.2, pp.210-218, 2017.

[55] M. Almeida, L. Han, M. Martin-Millan, et al., "Skeletal involution by age-associated oxidative stress and its acceleration by loss of sex steroids", *Journal of Biological Chemistry*, vol.282, no.37, pp.27285-27297, 2007.

[56] R.S. Balaban, S.Nemoto, and T. Finkel, "Mitochondria, oxidants, and aging", *Cell*, vol.120, no.4, pp.483-495, 2005.

[57] J.F. Passos, G. Nelson, C. Wang, et al., "Feedback between p21 and reactive oxygen production is necessary for cell senescence", *Molecular Systems Biology*, vol.6, pp.347, 2010.

CAPÍTULO 3

Extracellular vesicles: A new therapeutic strategy for joint conditions

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Biochemical Pharmacology (2018) **153:**134-146.

Extracellular vesicles: a new therapeutic strategy for joint conditions

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Abstract

Extracellular vesicles (EVs) are attracting increasing interest since they might represent a more convenient therapeutic tool with respect to their cells of origin. In the last years much time and effort have been expended to determine the biological properties of EVs from mesenchymal stem cells (MSCs) and other sources. The immunoregulatory, anti-inflammatory and regenerative properties of MSC EVs have been demonstrated in *in vitro* studies and animal models of rheumatoid arthritis or osteoarthritis. This cell-free approach has been proposed as a possible better alternative to MSC therapy in autoimmune conditions and tissue regeneration. In addition, EVs show great potential as biomarkers of disease or delivery systems for active molecules. The standardization of isolation and characterization methods is a key step for the development of EV research. A better understanding of EV mechanisms of action and efficacy is required to establish the potential therapeutic applications of this new approach in joint conditions.

Keywords: extracellular vesicles, mesenchymal stem cells, inflammation, immunomodulation, rheumatoid arthritis, osteoarthritis

Introduction

Extracellular vesicles (EVs) are actively secreted by cells and represent a mechanism for cell-to-cell signaling in physiological and pathophysiological responses [1,2]. These microparticles are usually classified based on the mode of biogenesis as microvesicles, exosomes, and apoptotic bodies [3]. Microvesicles and exosomes are both commonly found in extracellular fluids and represent the most described classes of EVs. Microvesicles are shedding vesicles between 50 nm and 1 μm in diameter generated by plasma membrane protrusions followed by fission of their membrane stalk [3,4]. Exosomes are formed as intraluminal vesicles in endosomal compartments called multivesicular bodies and they are released in an exocytic manner by fusion of these multivesicular endosomes with the plasma membrane. These EVs show a mean size of 40 to 100 nm in diameter and are enriched in endosome related proteins [4,5] whereas apoptotic bodies (50–5,000 nm in diameter) are released from fragmented apoptotic cells [3].

Joint conditions represent an important public health problem as they are a major cause of pain, functional limitation and physical disability. As a main example, rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial angiogenesis, hyperplasia of the synovial membrane and infiltration of immune cells besides cartilage damage and bone resorption [6]. RA is a systemic disease which can affect organs such as the lungs, heart and eyes and is associated with an increased risk of cardiovascular disease, infection, lymphoma, and reduced life expectancy [7,8]. The cause of RA is not yet fully understood, although autoimmune dysfunction plays a leading role in inflammation and joint damage, with a pre-rheumatoid phase preceding the onset of articular disease followed by established RA. Modification of the abnormal immune response by immune modulatory cells and other novel approaches represents an attractive possibility to achieve long-term tolerance and control of chronic inflammation [9].

Osteoarthritis (OA), the most prevalent joint condition in the elderly, is associated with progressive articular cartilage loss, low-grade synovitis and alterations in subchondral bone and periarticular tissues. There is an imbalance between anabolic and catabolic processes in the joint as well as a relevant contribution of mechanical stress and inflammatory mediators [10]. A number of risk factors are associated with OA, such as advancing age, obesity, and trauma that determine the progression of pathophysiological

events in joint tissues [11]. There is no effective treatment for OA although new therapies to stop disease progression or repair tissue damage are been investigated.

In the last years, the interest for the therapeutic applications of EVs has exponentially increased as these microparticles may reproduce the effects of parent cells with some advantages. In particular, EVs from mesenchymal stem cells (MSCs) provide a promising approach for immunomodulation and tissue regeneration. How these effects are mediated are not yet clear but EVs represent a novel strategy for future cell-free therapy of joint pathologies. Although these studies are at an early stage, the possible activity of EVs in joint conditions is of great interest and will be the focus of this review.

Table 1 Best established methods for the isolation of EVs [12–18].

Method	Principle	Scalability	Advantages	Challenges
Precipitation	Precipitant agent	Yes	Fast Simple	Low purity Protein contaminations Precipitant interferences
Differential ultracentrifugation	Size	No	Common EV subfractioning	EV aggregation Possible loss of function
Density gradient ultracentrifugation	Density	No	Common EV subfractioning High purity	Gradients may interfere with EVs' activity Possible loss of function Time consuming
Ultrafiltration	Size	Yes	High concentration	Aggressive Bias towards pressure-resisting EVs
Size exclusion chromatography	Size	Yes	High purity Removal of soluble proteins	Low yield Need of concentration
Immunoaffinity	EV phenotype	No	Fast High purity	Low yield Expensive Bias towards known markers-containing EVs

Isolation and characterization of extracellular vesicles

Current methods for isolating exosomes from biologic fluids include differential ultracentrifugation, density gradient centrifugation, size exclusion chromatography, polymer-based precipitation, filtration and immunoaffinity capture, as summarized in table 1. All of them have limitations such as co-isolation of contaminating materials, loss of EV components due to damaged membrane integrity during isolation or failure to completely isolate EV fractions. In particular, removal of serum proteins and lipoproteins

is problematic [12]. Depletion of the most abundant serum proteins such as albumin or immunoglobulins seems necessary to avoid biasing downstream analysis. Sample collection from different biological fluids should take into consideration possible sources of artefacts and variability [13]. Platelet removal, for example, is mandatory when working with blood, plasma or serum, as platelets release EVs upon activation in freeze-thaw cycles [14]. Additionally, serum used to supplement culture media must be previously EV depleted [15]. Therefore the presence of contaminants may influence the behavior of EV preparations leading to confusing effects on target cells. In addition, EVs from different sources can exhibit differences in composition or in non-specific component aggregation to their surface which can alter their physicochemical properties [16] and diverse EV subpopulations can be secreted by the same cell [17]. Consistency of pre-analytical procedures and report of complete experimental details have been recommended in order to get reproducible results [12].

There has been a great improvement of detection technologies during the past 20 years [18]. Quantification of EVs is usually performed by nanoparticle tracking analysis, tuneable resistive pulse sensing [19] or dynamic light scattering, and morphology confirmed by transmission electron microscopy, cryo-electron microscopy or atomic force microscopy [12]. Determinations of protein to lipid ratio, lipid bilayer order, and lipid composition may prove useful for quality control of EVs [20]. Western blotting or flow cytometry with fluorescent counting beads are normally used to detect EV protein markers [21]. The detection of specific markers would include CD63, CD9, and CD81 tetraspanins and endosome markers such as syntenin-1, ALG-2-interacting protein X (Alix) and tumor susceptibility gene 101 protein (TSG101), for exosomes [4,5,22]. Microvesicles can include cytoskeletal components (actin, actin-binding proteins (profilin-1, cofilin-1), myosin, tubulin), enzymes (alpha-enolase, pyruvate kinase, triosephosphate isomerase), membrane molecules (HLA-I, HLA-II antigens, Na^+/K^+ ATPase), proteins involved in vesicle biogenesis and trafficking (e.g. Ras-related proteins), lactadherin that binds to the phosphatidyl-serine surface of microvesicles, or clusterin (ApoJ), a protein involved in the clearance of apoptotic bodies and cell debris [21]. In addition, a set of components is cell-specific. Therefore, EVs from MSCs express on their surface MSC markers CD29, CD73, CD44 and CD105, as well as cell adhesion molecules and growth factor receptors. Inside EVs, a wide range of active molecules can be found such as cytokines, enzymes, nuclear receptors, miRNAs and other RNAs such

as transcription factor CP2/clock homolog, retinoblastoma-like-1, ubiquitin-related modifier-1 and interleukin-1 receptor antagonist [23].

Studies in 3T3-L1 mature adipocytes have shown a role for protein and lipid content in the characterization of large EVs (probably including microvesicles, with expression of β-actin and enrichment in endoplasmin and α-actinin-4), and small EV populations (sEVs, with expression of exosomal markers Alix, TSG101 and tetraspanins). The lipidomic analysis indicated cholesterol enrichment of sEVs, whereas large EVs were characterized by high amounts of externalized phosphatidylserine [24]. It has also been demonstrated the presence of two distinct subpopulations of exosomes (low density fractions exosomes and high density fractions exosomes). Both types express the exosomal markers Alix and TSG101 but differed in the presence of α-actinin-4, cyclin-Y (enriched in low density fraction exosomes) and ephrin type-A receptor 2 proteins (enriched in high density fractions exosomes) as well as in their RNA content [17]. As isolation methods based on different biogenesis pathways are still lacking, an universal nomenclature has been proposed based exclusively on size: large EVs pelleted at low speed, medium-sized EVs pelleted at intermediate speed, and sEVs pelleted at high speed. Among sEVs, further subcategories may be distinguished based on the presence or absence of different markers: a, enriched in CD63, CD9 and CD81 tetraspanins and endosome markers; b, devoid of CD63 and CD81 but enriched in CD9; and two groups not associated to the endosomal pathway: c, devoid of CD63/CD9/CD81; and d, enriched in extracellular matrix or serum-derived factors [22].

There is an increasing interest in the structural and functional biology of EVs. In addition to common components [25], these microparticles contain markers from the parent cells and therefore cell type specific protein, mRNA, miRNA, and lipid subsets have been identified which can be useful for diagnostic and therapeutic purposes. Interestingly, stress conditions or activation of intracellular signaling by mediators such as cytokines change EV composition and therefore the response of recipient cells [26-28]. The content of proteins, RNA and lipids has been investigated by high-throughput methods. Genomic DNA has also been detected in EVs although its function is unknown [28]. In addition to classical techniques, proteomic analyses of EVs can be performed by high-resolution and high-sensitivity mass spectrometry and high-resolution liquid chromatography mass-spectrometry-based approaches [29]. These techniques and gas chromatography coupled to mass spectrometry, provide information on the presence of lipid species and

metabolites in EVs [30]. EVs contain lipids in a bilayer membrane and also transport bioactive lipids and lipid related enzymes such as phospholipase A_2 and other enzymes involved in eicosanoid synthesis. Besides, EVs are enriched in cholesterol and sphingomyelin which can accumulate in recipient cells [31] as EVs may transfer lipids between cells for metabolism into bioactive mediators [32]. Metabolomic strategies have recently provided the characterization of EV metabolic activity [30]. Interestingly, high throughput transcriptomic studies have identified a wide range of mRNA and miRNA data sets based on microarray and next-generation sequencing analyses leading to a comprehensive data classification [33,34]. These EV components can be functional after transfer to cells [35]. Other RNA species within EVs include viral RNSs, Y-RNAs, fragments of tRNAs, small nuclear RNA, small nucleolar RNA, piwi-interacting RNAs, long non-coding RNAs and circular RNAs [28,36]. There are a wide range of studies on EV composition which are collected by three curated data repositories: ExoCarta [37], Vesiclepedia [3] and EVpedia [38], and functional enrichment analysis tools are also available [39].

Immunomodulatory effects of extracellular vesicles

A wide range of evidence indicates that EVs produced by both immune and non-immune cells can play an important role in the regulation of immunity (reviewed in [40,41]). Circulating endogenous EVs produced by different cell types contribute to the suppression of immune responses, either in an antigen-specific or a nonspecific manner. For instance, platelet-derived EVs can inhibit inflammatory responses due to the presence of 12-lipoxygenase which is transferred to mast cells to synthesize the pro-resolving mediator lipoxin A_4 [42]. Endothelial cell-derived EVs can suppress monocyte activation due to the transfer of miRNAs such as miR-10a able to target several components of the nuclear factor-κB (NF-κB) pathway, including interleukin-1 receptor-associated kinase 4 [43]. Some studies have suggested that antigen-specific immunosuppressive EVs from autologous plasma may be used to inhibit inflammation. Interestingly, intraarticular injection of exosomes obtained from autologous conditioned serum was safe and reduced pain and inflammatory markers in RA patients who do not respond well to conventional therapy [44]. In addition, blood-derived exosomes may be negative regulators of osteoclast formation in RA [45].

Oxidative stress plays an important role in the regulation of the immune response in arthritis [46]. In RA patients there is a significant elevation of surface thiols on circulating monocytes while the newly released EVs of isolated CD14+ cells from these patients have decreased thiol levels and enhanced peroxyredoxin 1 expression compared with healthy subjects. These results suggest that production of EVs by human monocytes may regulate oxidative stress in these cells [47]. It has been reported that macrophages release EVs containing Gla-rich protein which is a calcification inhibitor in articular tissues and a possible anti-inflammatory agent in chondrocytes, synoviocytes and monocytes/macrophages. This protein may link inflammation and calcification events in the joint and is able to inhibit the production of pro-inflammatory cytokines in macrophages [48].

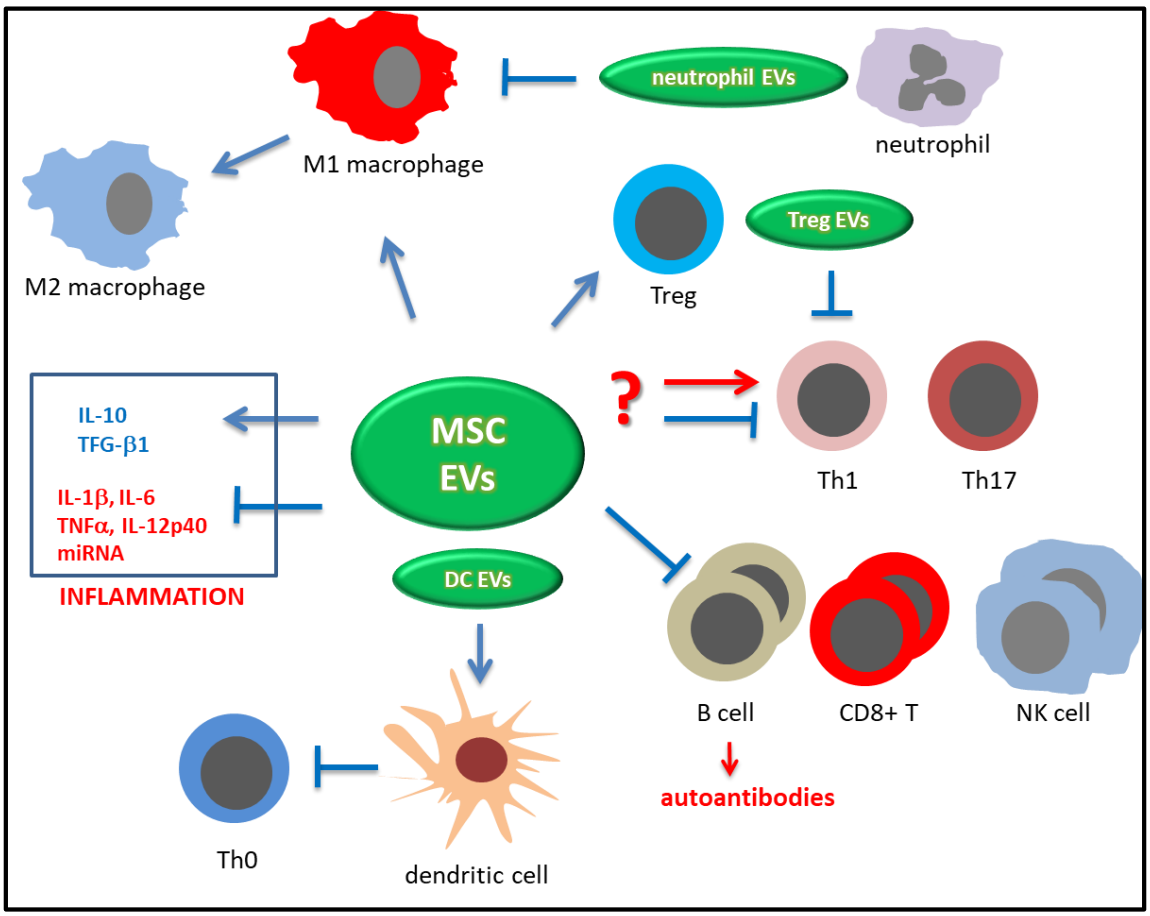

Fig. 1 Immunosuppressive effects of EVs from MSCs and other cell types. EVs from MSCs reduce the proliferation and differentiation of CD8+ T cells, B cells and NK cells while favor the differentiation of Treg cells and the switch of pro-inflammatory monocytes and macrophages (M1) to an anti-inflammatory phenotype (M2). The effect on CD4+ T cells has not been clearly demonstrated. Neutrophil EVs exhibit anti-inflammatory actions on macrophages. EVs from Treg cells inhibit Th1 cell proliferation. DC EVs can interact with T cells and APCs to alter their function. In inflammatory conditions, EVs inhibit the production of pro-inflammatory mediators and enhance that of anti-inflammatory and pro-resolution mediators in different cell types.

On the other hand, human neutrophils release EVs able to block inflammatory responses of macrophages and induce the release of transforming growth factor-β1 (TGF-β1) which can promote the resolution of the inflammatory response [49]. Neutrophil-derived microvesicles also exert chondroprotective actions *in vitro* and in murine models of inflammatory arthritis. It has been demonstrated that neutrophils migrate into inflamed joints to release microvesicles which penetrate into the cartilage. Neutrophil microvesicles require annexin A1 and its receptor formyl peptide receptor 2 to exert protective effects on chondrocytes which are mediated by TGF-β1 production, extracellular matrix deposition and inhibition of chondrocyte apoptosis. Interestingly, RA synovial fluids contain abundant neutrophil-derived microvesicles with a possible cartilage protecting role [50].

Dendritic cell (DC)-derived EVs deliver their content into the cytoplasm of acceptor DCs which could be a mechanism involved in fine-tuning of the immune response [51]. Nevertheless, These EVs are not only vehicles to deliver immunosuppressive factors from their parent cells as they exert antigen-specific effects which depend on the presence of molecules such as MHC class II and B7. Distal therapeutic effects were also observed after local administration of DC EVs suggesting that they may act by interacting with endogenous immune cells at the membrane level or by transfer of proteins and RNAs leading to an immunosuppressive and anti-inflammatory behavior of these cells [44]. Exosomes from immature DC may be partially immunosuppressive [52] and they can be modified to enhance this property. It has been suggested that IDO expression in DCs modifies exosomes to render them tolerogenic. Therefore, exosomes derived from DCs overexpressing indoleamine 2,3-dioxygenase (IDO) have an anti-inflammatory effect in collagen-induced arthritis (CIA) and delayed-type hypersensitivity murine models. These exosomes may directly interact with T cells and other antigen-presenting cells (APCs) to alter their function which was partially dependent on B7 costimulatory molecules [53]. In the CIA model, intravenous administration of exosomes derived from DCs expressing interleukin(IL)-10, DCs expressing IL-4 or DCs expressing FasL-effectively inhibited arthritis [54,55]. Injected exosomes are internalized by CD11c+ cells at the site of injection and in the draining lymph node. Local administration of exosomes was also able to inhibit the inflammation of murine delayed-type hypersensitivity in both the treated and the untreated distal paws in a MHC class II dependent and MHC class I independent manner [55].

The ability of Treg cells to release exosomes is required to inhibit Th1 cell proliferation *in vivo* and prevent systemic disease. It has been reported that the miRNA content of exosomes play an important role in this inhibitory effect. Therefore, let-7d is transferred to Th1 cells and mediates the suppression of Th1 cell proliferation and interferon-γ (IFN-γ) secretion [56]. The transfer of the dominant negative form of inhibitor of NF-κB kinase 2 (IKK2) has been used to give rise to immature CD4+CD25-Treg cells (dnIKK2-Treg). These cells release EVs containing specific miRNAs and inducible nitric oxide (NO) synthase which are delivered into target cells leading to block of cell cycle progression and induction of apoptosis. In addition, dnIKK2-Treg-EV-exposed T cells can be converted into regulatory cells [57].

Cell infiltration and the imbalance between cell proliferation and cell death contribute to pathological changes in RA. In particular, the resistance of synovial lymphocytes, macrophages and fibroblasts to apoptosis may play a role in the chronification of arthritis [58]. The bioactive death ligands FasL and APO2L/TRAIL are stored inside human T cells and secreted associated with EVs upon cell activation [59]. Interestingly, the number of EVs containing APO2L/TRAIL in synovial fluid is very low in RA patients and the persistence of activated T lymphocytes has been related to the resistance to Fas/CD95 and the inefficient secretion of EVs containing bioactive FasL and APO2L/TRAIL [60]. Therefore, EVs expressing APO2L/TRAIL may be a therapeutic approach for RA which has been explored in preclinical models. Intraarticular injection of artificial lipid vesicles resembling natural EVs with bound APO2L/TRAIL exerted anti-inflammatory effects and inhibited synovial hyperplasia in a model of antigen-induced arthritis in rabbits [61].

EVs from other sources may be of interest for their immunoregulatory properties. For instance, bovine milk contains EVs expressing CD63 and immunoregulatory miRNAs (miR-30a, -223, -92a). Oral administration of bovine milk derived EVs delayed the onset of CIA and diminished cartilage pathology, bone marrow inflammation and serum monocyte chemoattractant protein-1, IL-6 and anticollagen IgG2a levels, accompanied by reduced splenic Th1 (Tbet) and Th17 (RORγt) mRNA [62].

MSC EVs have been shown to reduce inflammation, regulate immune responses and facilitate tissue regeneration [63]. There are complex interactions between MSCs and immune cells that may help to understand their immunomodulatory properties. The effects of MSCs are mediated by cell-to-cell contact and paracrine mechanisms due to the production of soluble molecules and EVs released into the extracellular milieu. The

importance of cell-to-cell contact in immunosuppression by MSCs has been shown in different studies as well as the role of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 [64,65]. MSCs have been reported to secrete a wide range of molecules such as purines, bone morphogenetic proteins (BMPs), CD274, CCL2, connexin 43, IDO [66], prostaglandin E_2 [67], IL-6, IL-10, NO [68], heme oxygenase-1 [69], tumor necrosis factor-inducible gene-6 (TSG-6) [70], leukemia inhibitory factor (LIF), CD95/CD95 ligand, galectins, human leukocyte antigen-G5 (HLA-G5) [71], and growth factors such as TGF-β1 [68], hepatic growth factor (HGF) [72], vascular endothelial growth factor (VEGF), platelet-derived growth factor, fibroblast growth factor (FGF), etc. [73]. Other ways of cellular communication between MSCs and immune cells include the bidirectional exchange of cytoplasmic components mediated by tunneling nanotubes derived from human T cells [74] and the transfer of EVs.

Cellular therapy with stem cells showed a low engraftment and poor survival leading to the demonstration that MSCs act through paracrine effects in animal models of ischemic heart disease and acute kidney injury [75-79]. Therefore, it was demonstrated that only the fraction of the conditioned medium (CM) containing products >1000 kDa (100-220 nm) provided cardioprotection in a mouse model of ischemia and reperfusion injury [80] leading to the confirmation that protective effects of MSC secretome depended on the presence of EVs [80-82]. In addition, EVs exert a modulating role on the effects of soluble mediators [83].

The beneficial effects of MSCs in glucocorticoid-refractory graft-versus-host disease in human patients have been related to immune response-modulating factors secreted by these cells and identified as EVs [84]. Similarly, EVs from umbilical cord (UC)-MSCs ameliorated the inflammatory immune reaction and kidney function in grade III-IV chronic kidney disease patients [85]. Phase I clinical studies with EVs have revealed a low toxicity and stability in plasma and different clinical studies have tested their potential in wound healing [86], hair regeneration [87], acne scars and skin rejuvenation [JSPH2012-082], type-1 diabetes [NCT02138331], the development of vaccines for different types of cancer or as vehicles for drug delivery to cells [88].

The degree of EV-mediated immunomodulation seems to be proportional to the ability of different immune cells to uptake these microparticles [89,90] leading to the inhibition of proliferation and differentiation processes [91]. EVs from MSCs may exert the strongest

immunomodulatory effects on B cells compared with other lymphocyte subsets which may depend on the ability of B cells to incorporate EVs. Therefore, EVs from MSCs inhibit the proliferation of B cells and also of NK cells [89]. Nevertheless, the role on T cells has not been clearly demonstrated. It has been reported that exosomes from adipose-derived MSCs (AMSCs) exert an inhibitory effect on proliferation, differentiation and activation of T cells [92]. In contrast, microvesicles from bone marrow (BM)-MSCs have been shown to exert a lower immunomodulatory effect on T-cell proliferation compared with the parent cells [93]. Other reports failed to demonstrate any effect on lymphocyte proliferation by EVs from MSCs [74]. In another study, BM-MSC EVs exhibited *in vitro* immunomodulatory effects on T cells but they were different from those of their parent cells [94]. Immunosuppressive effects of BM-MSCs can be enhanced by priming with IFN-γ and tumor necrosis factor-α (TNFα) which leads to higher ICAM-1 expression and internalization of EVs by immune cells. In addition, primed EVs enhance the immunosuppressive ability of resting BM-MSCs towards T cells, which may be mediated by IDO increase [89]. Another report has indicated the possible contribution of cyclooxygenase-2 and different miRNAs to the immunosuppressive effects of cytokine-stimulated BM-MSCs [95]. Microvesicles and exosomes from murine MSCs have been shown to inhibit the proliferation of CD8+ T cells and the proliferation and activation of B cells. In addition, both types of EVs increased the Treg population but were without effect on CD4+ IFNγ+ T cells [96].

EVs released by MSCs are efficiently internalized by macrophages and induce proliferation and the transition of pro-inflammatory macrophages to an anti-inflammatory and pro-resolving M2 phenotype [97]. It was demonstrated that microvesicles from murine AMSCs were quickly incorporated into the intracytoplasmic region of M1-macrophages and promoted a M2-like phenotype and the reduction of pro-inflammatory miR-21 and miR-155. These results were confirmed *in vivo* in an experimental model of acute peritonitis [98]. Also, M2 polarization was induced by MSC EVs in mouse or human monocytes which in turn polarized activated CD4+ T cells to CD4+CD25+FoxP3+ Treg cells [99]. In contrast, EVs from UC-MSCs did not polarize monocytes [100]. Figure 1 shows a summary of the immunosuppressive effects of EVs.

Few studies on the *in vivo* effects of EVs in arthritis models have been published. It has been reported that administration of EVs from murine MSCs ameliorated the symptoms in the mouse CIA model of RA [96]. In bovine serum albumin-induced synovitis in pigs,

intraarticular administration of EVs from porcine BM-MSCs exerted anti-inflammatory effects with reductions in synovial lymphocytes count and TNFα expression. These EVs efficiently counteracted the antigen-driven T cell response and may represent a therapeutic strategy for the treatment of T cell mediated diseases such as RA [101].

Transfer of EVs components can play an important role in the effects of these microparticles. MSC EVs include a cargo of immunomodulatory proteins which may act in a synergistic manner [102]. These microparticles thus induce high levels of anti-inflammatory TGF-β1 and IL-10 [103,104], and inhibit pro-inflammatory IL-1β, IL-6, TNF-α and IL-12p40 [99]. Microvesicles derived from mouse BM-MSCs express regulatory molecules present in parent cells such as programmed death-ligand 1 (PD-L1), galectin-1 (Gal-1) and TGF-β1 which confer tolerogenic properties to these microparticles [105]. Interestingly, PD-L1 contributes to the development of inducible T regulatory (iTreg) cells [106] while Gal-1 has been shown to induce growth arrest and apoptosis of activated T cells and contribute to the promotion and generation of Treg cells [107,108]. Therefore, Gal-1 gene therapy or protein administration to DBA/1 mice inhibited clinical and histological manifestations of arthritis in the CIA model [109]. The results of these studies support the interest of EVs in the treatment of chronic inflammatory and autoimmune disorders. In addition, microvesicles from MSCs contain ribonucleoproteins involved in the intracellular traffic of RNAs as well as selective miRNAs which may be transferred to target cells [110] and likely exert immunomodulatory effects in arthritic diseases (reviewed in [111]). Apart from the presence and properties of endogenous miRNAs, loading of EVs with miRNAs or anti-miRs may be an interesting approach to enhance the immunoregulatory activity of these microparticles in chronic inflammatory conditions [42].

Regenerative properties of extracellular vesicles in joint conditions

Joint conditions are important targets of MSC therapy mainly to treat chondral and/or bone lesions and defects resulting from injury or trauma, or in OA. In the last years, the possible applications of MSCs in cartilage repair used alone or combined with biomaterials have been extensively explored. MSCs are injected into the joint space, or implanted in a scaffold matrix or as tissue engineered constructs in order to create a favorable microenvironment for tissue repair (for review see ref. [112]). Stem cells are

capable of selectively homing to injured tissues and differentiating into several types of cells to repair the lesion and improve the affected function. Humoral mediators produced by injured tissue would be chemotactic for stem cells, they also stimulate local proliferation of endogenous or exogenous stem cells or could be a signaling mechanism to expand the pool of bone marrow progenitor cells in response to tissue injury [113]. Nevertheless, it has been reported that chondrogenesis in 3D culture generates constructs whose mechanical properties are inferior to constructs formed with chondrocytes [114] leading to studies on different strategies to improve the chondrogenic potential of MSCs [115].

Cell differentiation and engraftment would not be the sole mechanisms for tissue regeneration as transplanted cells become fewer and disappear soon after transplantation [115]. Additionally, MSCs exhibit a variety of trophic activities relevant to musculoskeletal therapy and can promote chondrogenesis, osteogenesis, musculogenesis, tenogenesis, angiogenesis and neurogenesis (reviewed in [73]). The efficacy of MSC therapies in joint repair has been demonstrated in many animal models and clinical studies [116-126].

The effectiveness of many MSC-based therapies in tissue repair has been attributed to the paracrine secretion of these cells as only a small percentage of the MSC populations injected into the joint actually remain at the site of injury (reviewed in [8]). The MSC secretome would promote tissue repair by modulating the local microenvironment and supporting growth and activity of local cells. Nevertheless, the composition of MSC secretome is quite complex and varies depending on the microenvironment of cells [127]. For instance, MSCs have a differential response to synovial fluid from early- versus late-stage OA, with a higher secretion of CXCL8, IL6 and CCL2 in the first case [128]. Accordingly, pretreatment of MSCs with different factors can improve the release of immunomodulating or regenerating mediators [129] as it has been shown by priming the parent MSCs with lipopolysaccharide [130].

The CM of MSCs contains a wide range of cytokines, chemokines, hormones, lipid mediators, cytokines, growth factors and extracellular matrix components which can mediate tissue healing. The regenerative properties of CM from MSCs have been explored in many different tissues (reviewed in [88]). Therefore, MSC CM can regenerate bone through mobilization of endogenous stem cells, angiogenesis and osteogenesis [131] and promote periodontal tissue regeneration [132] and healing of bisphosphonate-related

osteonecrosis of the jaw in rats [133]. Interestingly, the therapeutic efficacy of human BM-MSCs CM was demonstrated in a human clinical study. This CM containing insulin-like growth factor-1, VEGF, TGF-β1 and HGF, in beta-tricalcium phosphate or an atelocollagen sponge, regenerated alveolar bone [134].

Treatment of OA chondrocytes or synovial cells with CM from BM-MSCs or AMSCs in an inflammatory milieu inhibits the production of inflammatory and catabolic agents [9,10]. In this respect, we have reported that CM from AMSCs exert protective effects in OA chondrocytes [20,21]. Therefore, factors produced by MSCs can enhance the anabolic properties of a wide range of cells such as chondrocytes, chondrocyte progenitor cells, cartilage-derived stem/progenitor cells, synovium-resident multipotent progenitor cells, osteoblasts/osteoclasts/resident MSCs in subchondral bone and chondrogenic cells within the infrapatellar fat pad [73].

The EVs present in CM show a great potential in the regeneration of joint tissues to replace stem cell-based therapy. EVs secreted by hMSC carry hyaluronan on their surface which is able to interact with proteins and proteoglycans of extracellular matrix to maintain tissue homeostasis, and contribute to extracellular matrix remodeling and tissue healing [135,136]. EVs express adhesion molecules to bind to and interact with cells, but they are also able to bind to extracellular matrix components. EVs from some cell types contain extracellular matrix regulatory proteins involved in re-structuring, cytokine release, angiogenesis and cell migration as well as lysyl oxidases which crosslink collagens and elastin [137,138]. In particular, exogenous lysyl oxidases have been shown to be useful in cartilage integration problems [139]. Interestingly, EVs from different cell types are able to transfer the mRNA of growth factors and their receptors to tissue cells to initiate tissue repair responses [140,141].

Treatments with MSC EVs are able to reproduce the main actions of CM suggesting that these microparticles are relevant mediators. EVs from naïve or genetically modified MSCs may be used to improve the regenerative properties of these cells as they can modulate the microenvironment of damaged cartilage to promote repair or to enhance the chondrogenic ability of these cells [124]. Different scaffolds have been investigated to retain MSC EVs and promote cartilage repair. One of them is a photoinduced imine crosslinking hydrogel glue with excellent biocompatibility and integration with cartilage matrix which has been tested to prepare an acellular tissue patch for cartilage regeneration [142].

Interestingly, OA chondrocytes internalize EVs from BM-MSCs leading to the upregulation of aggrecan and type II collagen. In addition, gene expression of IL-1, IL-6, IL-8 and IL-17 as well as collagenase activity induced by TNFα were significantly reduced [143]. It has also been reported that EVs from mouse BM-MSC exert anti-apoptotic effects in chondrocytes and immunosuppressive effects in macrophages. *In vivo* administration of these EVs partly protected cartilage and bone in the murine collagenase model of OA [144]. We have provided evidence that microvesicles and exosomes from human AMSC CM exert anti-inflammatory and protective effects in OA osteoblasts [145] and chondrocytes (our unpublished results) *in vitro*. Anti-inflammatory and chondroprotective effects of EVs derived from AMSCs have also been described in murine cells [146].

The release of pro-inflammatory mediators and reactive oxygen species can result in mitochondrial changes, inflammation, oxidative stress and DNA alterations which can induce premature senescence [37]. We have recently shown that microvesicles and exosomes from human AMSCs reduce the production of inflammatory mediators, mitochondrial membrane alterations and oxidative stress in OA osteoblasts which results in the down-regulation of cell senescence [145] (Figure 2).

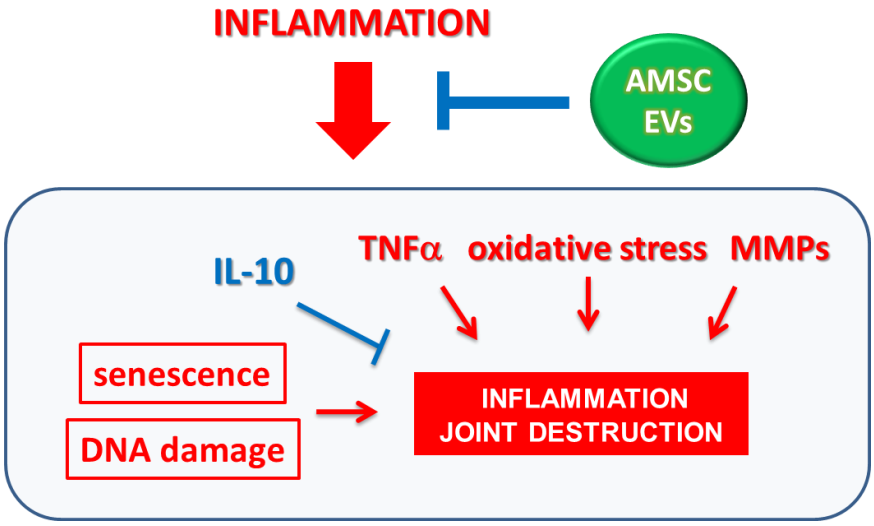

Fig. 2 Joint protective effects of AMSC EVs. In OA osteoblasts or chondrocytes subjected to inflammatory conditions, EVs reduce the production of pro-inflammatory and catabolic mediators as well as the induction of DNA damage and cell senescence while the production of the anti-inflammatory cytokine IL-10 is enhanced.

Different studies have demonstrated that EVs enhance skeletal muscle [147], bone [148] and cartilage [142] regeneration. Administration of AMSC EVs in a model of skeletal muscle injury reduced the inflammatory response and accelerated the muscle regeneration process [97]. BM-MSC-derived EVs led to bone formation in calvarial bone defects with an essential role for miR-196a in the regulation of osteoblastic differentiation [148]. Injection of exosomes from human induced pluripotent stem cell-derived MSCs (iPS-MSCs) by intravenous route prevented osteonecrosis induced by steroid in rats. This treatment activated the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway on endothelial cells leading to local angiogenesis [149]. Protective effects of these type of exosomes were also observed in an ovariectomized rat model [150] and, in combination with a tricalcium phosphate scaffold, in rat calvarial bone defect. *In vitro* studies showed that these exosomes can be internalized into BM-MSCs and enhanced the proliferation, migration, and osteogenic differentiation of these cells. Activation of the PI3K/Akt signaling pathway by exosomes likely plays an important role in these effects [151].

Intraarticular injection of exosomes obtained from human ESCs has been shown to completely restore cartilage and subchondral bone in a model of osteochondral defects in rats [152]. In another study, exosomes secreted by human synovial MSCs were internalized by human chondrocytes *in vitro* and induced proliferation and migration but reduced extracellular matrix production. These effects were due to the high Wnt5a and Wnt5b expression in these cells which activated YAP and led to the suppression of SOX9 expression. *In vivo* intraarticular injection of exosomes resulted in a weak protective effect in the rat OA model established by transecting the medial collateral ligament and the medial meniscus [153]. In another OA model in mice injected with collagenase, intraarticular administration of human synovial MSC exosomes significantly attenuated OA progression. In the same model, injection of exosomes from human iPS-MSCs had a superior therapeutic effect. Therefore, these cells may be a better source of exosomes for cartilage repair with other advantages as they can be induced from patient-specific adult somatic cells such as peripheral blood cells without an invasive harvesting and with a high yield. In addition, autologous cells can be used overcoming ethical issues and immune activation [154].

Some treatments using EVs from other sources may also be useful in OA. As an example, EVs from autologous conditioned serum have been shown to protect human OA cartilage from glycosaminoglycan loss in basal conditions and in the presence of IL-1β [155].

There is an ongoing observational study that evaluates the characteristics of autologous platelet-rich plasma (PRP) in the therapy and treatment of musculoskeletal pain and OA. This study will test the hypothesis that PRP characteristics, such as platelet and microparticle content and composition can be predictive for clinical outcome for PRP treatments (NCT02726464).

Further studies are necessary to establish the mechanisms underlying the regenerative effects of EVs but these microparticles contain many regulatory molecules that may be transferred to target cells and contribute to their biological effects. It has been suggested that EVs delivery to damaged tissue may contribute to epigenetic reprogramming of target cells [156,157]. MSC EVs repair ability can depend on the restoration of cartilage homeostasis. In OA, there is chondrocyte loss or cellular senescence induced by abnormal mechanical stress, inflammation, oxidative stress and mitochondrial dysfunction [158,159]. EVs may transfer to chondrocytes glycolytic enzymes such as phosphoglucokinase and pyruvate kinase, and ATP generating enzymes such as adenylate kinase and nucleoside-diphosphate kinase that may compensate the reduced mitochondrial ATP production in OA chondrocytes. In addition, MSC EVs contain CD73 which is able to convert the extracellular ATP released by injured tissues to adenosine. It is known that EVs induce cell proliferation through adenosine-mediated phosphorylation of extracellular signal-regulated kinase (ERK)-1/2 and Akt [160].

On the other hand, the regulation of immune cells and the pro-inflammatory environment plays an important role in tissue regeneration. M1 polarized synovial macrophages from OA patients secrete factors inhibiting MSC chondrogenesis [161] whereas MSCs can induce M2 polarization which reduce inflammation and improve cartilaginous tissue regeneration [162]. As EVs can exert immunomodulatory and anti-inflammatory effects, it is likely that these properties contribute to joint protection and repair.

MSC-derived EVs contain a wide range of miRNAs which may facilitate intercellular communication and contribute to different phases of the healing process [97,163]. Many of these miRNAs are involved in signal transduction, cartilage metabolism and OA progression. For instance, mir-23b is a mediator of chondrocyte differentiation of human MSCs [164] and miR-221 and miR-92a may contribute to the regulation of cell proliferation and differentiation of chondroprogenitor cells [165-167]. It is also known that key catabolic enzymes such as aggrecanase-2 and metalloproteinase-13 are the target of miR-125b and miR-320, respectively [168,169]. The expression of miR-320 is reduced

in OA cartilage compared with normal cartilage. This miRNA regulates chondrogenesis and IL-1β-stimulated catabolic effects in mouse chondrocytes [169]. Another miRNA with therapeutic potential in OA is miR-140, which regulates cartilage homeostasis and development [170]. Interestingly, modification of synovial MSCs to overexpress miR-140-5p improved EV properties *in vitro* and *in vivo* leading to a significant inhibition of cartilage degradation in a surgical model of knee OA in rats [153]. All together these data support the interest of EVs containing miRNAs with beneficial effects on joint metabolism to develop potential therapeutic approaches in OA.

Extracellular vesicles in the physiopathology of joint conditions

EVs mediate cell communication in pathological states and may act as signaling structures involved in the induction and amplification of immunity and inflammation. Therefore, some EVs can play a pathogenic role in joint conditions (for extensive reviews see refs. [171-176]). EVs may exert different roles in inflammation depending on the cell source, cell target and the environment where they can be influenced by multiple factors. Some studies have revealed that EVs are a component for autocrine and/or paracrine stimulation although they may also initiate counter-regulatory mechanisms which potentially contribute to the resolution of inflammation. In this context, EVs released by activated human monocytes have been shown to activate NF-κB and production of cytokines and oxygen radicals which may lead to the amplification of the inflammatory response. At the same time, these EVs are able to enhance PPAR-γ expression which is involved in the control of inflammation [177]. It is also known that EVs from T cells can modulate the effects of TNFα, suggesting a cross-talk between cytokines and EVs. Besides promoting the induction of inflammation, treatment with EVs significantly upregulated a number of anti-inflammatory genes [83].

The stimulation of immune responses by EVs in certain situations can initiate or exacerbate autoimmune diseases. EVs may exert immunostimulatory effects by a number of mechanisms. EVs express different molecules of the parent cells and may transfer antigens, MHC molecules and costimulatory molecules to immune cells. As an example, DCs produce EV-associated MHC class I complexes which are transferred to other naive DCs for efficient CD8+ T cell priming which can be viewed as an amplification process for DC-mediated CTL responses [178]. Similarly, EVs from both human and murine B

lymphocytes are able to induce antigen-specific MHC class II-restricted T cell responses. These studies support the view that EVs produced by APCs may act as vehicles for MHC class II-peptide complexes involved in maintenance of long-term T cell memory or T cell tolerance [179].

Synovial EVs formed in an inflammatory environment may stimulate articular cells to release more inflammatory mediators and degradative enzymes and thus contribute to articular damage [27,180]. Some EVs can also contain degradative enzymes and therefore EVs released by rheumatoid synovial fibroblasts degrade aggrecan in a tissue inhibitor of metalloproteinase-3-sensitive manner which may facilitate cell invasion through aggrecan-rich extracellular matrices [181]. Similarly, hexosaminidase activity is found to be associated with RA synovial fibroblast-derived EVs [180]. On the other hand, it has been reported that EVs released by chondrocytes contribute to pathologic mineralization of cartilage in musculoskeletal pathologies such as OA [182] and their secretion is regulated by autophagy [183].

More studies are necessary to dissect EV signaling pathways and molecular mechanisms in the physiopathology of joint conditions. As EVs produced by some cell types may be mediators of the pathophysiological changes that occur in the joint environment, a therapeutic strategy may be the inhibition of production and release, modification of harmful content or elimination of microparticles contributing to pathological processes [184].

Extracellular vesicles as biomarkers of joint disease

There is a considerable interest in identifying noninvasive specific biomarkers which may reflect the alterations in joint tissues. At present, prognostic tools especially for OA and spondyloarthritis are still lacking. Early identification of predictive markers is crucial to address the risk, the presence, the evolution and the response to treatment in chronic joint conditions [185]. The release of EVs into the extracellular space allows to examine them in body fluids as novel candidates for disease biomarkers to use in diagnosis, prognosis and treatment. Of note, in situations where the same biomarker molecules can be indicative of more than one condition, EVs would be the method of choice to trace the cell type causing the alteration. These microparticles can be immuno-isolated based on recognition of a significantly enriched protein on the membrane surface [186].

Serum EVs are enhanced in RA. In particular, endothelial EVs have a deleterious effect on endothelial cell function and may be a marker of vascular damage [187] while platelet-derived EVs levels may be related to disease activity [188]. Nevertheless, circulating EVs exposing complement components, C reactive protein or serum amyloid-P are elevated in early active RA although effective drug treatments do not decrease their levels suggesting a limited value as biomarkers [189].

Other reports indicate that serum EVs may be useful as additional markers of disease activity in patients with RA. For instance, differences in EV levels of amyloid A and lymphatic vessel endothelial hyaluronic acid receptor-1 have been found between the clinical remission and non-clinical remission groups [190]. In addition, high expression of Hotair has been demonstrated in blood mononuclear cells and serum EVs of RA patients whereas a lower level of Hotair was detected in differentiated osteoclasts and rheumatoid synoviocytes [191]. Platelet EVs are also elevated in RA and other inflammatory arthritis synovial fluid compared with OA and may play a role in the amplification of the inflammatory process. In this respect, collagen receptor glycoprotein VI has been identified as a key trigger for platelet EV generation in arthritis [192].

Synovial EVs contain citrullinated proteins, which are known autoantigens and biomarkers in RA [193]. In synovial fluid from RA patients, the number of microvesicles positive for receptor activator of NF-κB and its ligand are increased as well as CD3+ and CD8+ microvesicles which might reflect a locally enhanced activation of CD8+ T cells [21]. In addition, CD4+ T-cell-derived CD161+CD39+ and CD39+CD73+ EVs in synovial fluid have been recently proposed as reciprocal biomarkers for RA [194].

Differences in miRNA expression in EVs may lead to propose new biomarkers in joint conditions [174,195]. In the last years many studies have focused on circulating miRNAs as biomarkers of disease which represent an important part of EV composition (reviewed in [175]). The changes in synovial fluid-derived EV miRNA with joint alterations provide a unique opportunity to discover candidate biomarkers. Interestingly, studies of miRNA expression in synovial fluid EVs from OA patients have shown sex specific changes. Therefore, in female patients, miR16-2-3p was upregulated and miR26a-5p, miR146a-5p and miR-6821-5p were downregulated while in male patients, miR-6878-3p was downregulated and miR-210-5p was upregulated. These results also suggested that estrogen might play an important role in EV derived miRNA [196]. Therefore, a gender

dimension should be considered in the investigation of specific biomarkers for joint conditions.

Challenges in EV research

Several nomenclature and methodological challenges have raised concerns among the community about the reproducibility and comparability of the different reports published in recent years. In particular, the disparity of isolation and characterization approaches, and the lack of unified nomenclature and handling criteria are hindering the understanding of EVs biological functions [18]. In that regard, organizations such as the International Society for Extracellular Vesicles (ISEV) have published guidelines in an increasing effort to integrate the currently accepted isolation and characterization methods [18,197].

Vesicles shed from the cell plasma membrane are often called microvesicles, microparticles or ectosomes, with a sized ranged between 50-100 nm to even few micrometers depending on the author's criteria and the isolation method. Small vesicles secreted from multivesicular endosomal bodies are usually called exosomes and classically considered to be under 150 nm in diameter, but most common isolation procedures based on the use of 200-nm pore filters and ultracentrifugation, isolate mixed EV populations. In practice, EV classification is not clear-cut as microparticles exhibit overlapping similarities in size, morphology, density and protein markers of both endosome and plasma membrane [16] and even the presence of different subpopulations within the same EVs class has been demonstrated [17,22]. In addition, a single cell can release EVs with differences in size, biogenesis and content which can vary depending on the cell type and its physiologic state [198].

From upstream sample handling to isolation and characterization, there is presently no single standardized method to universally obtain pure EV products. Generally, a highly pure EV isolate is obtained at the expense of therapeutic potency, yield, cost and/or scalability. These considerations are of critical importance when dealing with EVs as therapeutic agents, as industrial scale production must deliver an acceptable compromise between purity, activity and cost [199]. Currently, characterization efforts have focused on physical properties such as size and concentration, and vesicular content in terms of protein, lipid and nucleic acid composition. As the smallest EVs reach sizes of 50 nm or

even less, current size analysis methods struggle to reach this detection limit, making comparisons of different concentrations difficult and statistically compromised.

Vesicular cargo includes proteins, RNA, DNA, lipids and metabolites, and may be inside EVs or on their surface. Subvesicular localization must be considered during characterization procedures to avoid artefacts and false positives. Importantly, the isolation method severely impacts the purity of EVs and therefore the omics profiles [200] and possible EV applications. However, as EV isolates contain disparate populations, current data should be considered as an average of the RNA content of all EV subpopulations. Additionally, most biofluids contain potential contaminants such as RNA- and miRNA-carrying proteins, making analyses difficult to decipher even after treatment with RNAses [201].

It is crucial to improve the methods to isolate and characterize the different EV types. This issue is a source of confusion leading to contradictory results but it is also the first necessary step for studies of pharmacological activity and therapeutic efficacy. As many factors can influence the reproducibility of effects, different steps need to be taken to assure homogenous EV preparations and guarantee their efficacy and safety. In the last years, the International Society for Extracellular Vesicles (ISEV) has released position papers and the Minimal Information for Studies on EVs (MISEV) to help researchers overcome these problems. Furthermore, to increase reproducibility and transparency of EV methodologies, the EV-TRACK knowledgebase has been recently developed [202]. Strict standardized protocols must be implemented to effectively control all aspects of EV production and application, from culture of source cells to medicinal product preparation and administration. Qualitative and quantitative EV technologies need to be thoroughly validated. New technologies may help to advance this research field. For instance, EV uptake can be determined at single cell level using the Cre reporter methodology or bioluminescence methods can be employed to determine EV release and uptake and new-omics approaches have been incorporated to improve the knowledge of molecular EV components [30]. In addition, there is a need for normalization and control in sample collection and methods for keeping and transporting EV samples. All these points are essential to detect relevant differences between health and disease in clinical studies. It is not surprising that results found in the literature showed EV clinical studies of small populations with small portion of large effect size. Improved methodologies and study

design are needed including larger numbers of samples in order to determine whether there is an effect at the population level [203].

Human EV-based therapeutics is subjected to the regulatory frameworks of biological medicinal products covering preclinical development, quality aspects, non-clinical safety requirements and the clinical testing. In the context of EV-related therapies and their approval, a complete *in vitro* and *in vivo* testing must be outlined. This should at least include assays to identify and characterize the components of the EV isolate (molecular fingerprinting), potency assays to quantify the EV-mediated therapeutic effect, and functional tests to determine their mechanisms of action as well as pharmacokinetic and toxicology studies. In the particular case of EV research, issues such as localization of molecules —inside the vesicle, embedded in the membrane, or associated outside— and mechanisms of cell-EV interaction —mainly vesicle internalization or plasma membrane receptor signaling— must be carefully taken into consideration for a thorough pharmacological validation [199].

For clinical application, compliance with safety standards related to inadvertent microbial and viral contamination and GxP standards (Good Manufacturing/Good Laboratory/Good Distribution/Good Clinical/Good Scientific Practice or GMP/GLP/GDP/GCP/GSP) is necessary for the production and quality control [1]. As a further step, one important hurdle is the ability to produce consistent products on a large scale. There is a need of developing clinical-grade robust and stable manufacturing processes.

Perspectives

Interest in cell-derived EVs has exponentially increased due to their proposed contribution to homeostasis and disease, and their potential as future therapeutic and diagnostic tools. In particular, EVs have recently received a great deal of attention as a possible better alternative to MSC therapy in autoimmune conditions and tissue regeneration. The induction of immunological reset by MSC EVs has become an attractive possibility in RA and other autoimmune conditions. while the use of EVs for joint repair and OA could potentially be a better cost-effective therapy compared with MSC administration [160].

EVs offer the possibility to develop cell-free therapeutic approaches with less regulatory obstacles and clinical risks associated to cell therapies. Besides, they may have potential advantages in biomanufacturing, storing and distribution and may represent a more reproducible therapeutic tool [90,94]. EVs contain many biomolecules from the parent cells and can have advantages compared with cell therapy, as injected cells may die or fail to fully home into the lesion while EVs injection allows for a more precise dosing schedule and a better control of treatment or suspension of administration. The use of EVs may also eliminate problems such as blood vessels occlusion and generation of altered cell phenotypes [160,204].

Compared with MSCs, which produce different molecules according to the microenvironment leading to complex interactions or can exhibit opposite effects depending on the stimulus used to trigger immune cells [205], EVs may lead to results less dependent on the environment and more predictable. The content of these microparticles is protected from enzyme degradation, and this natural mechanism can be used to deliver active molecules to cells. In this respect, EVs are less likely to alter target cells than artificial nanoparticles. The small size may be an advantage in relation with the selection of administration routes in comparison with cell therapy. In addition, their bi-lipid layer vesicular structure is membrane permeable and their surface proteins may confer targeting ability due to their affinity for specific cell membranes or extracellular matrix in diseased tissues [79,157,206].

EVs have a lower immunogenic potential compared with cells [204] and thus allogeneic EVs have been reported to be safe and may be an appropriate source for large-scale production [160] in preclinical studies and clinical applications. In this respect, technological advances can improve large-scale preparation of EVs. For instance, recent studies have demonstrated that microvesicle production by MSCs can be amplified using a 3-D bioprocessing method keeping the biological activity of these microparticles [207].

Modification of EVs may improve their properties to regulate different processes. Therefore, the parent cells could be primed or genetically modified and then expanded in order to produce modified EVs e.g. without histocompatibility antigens to minimize the possibility of immune reactions, expressing relevant proteins, lipids or RNA to maximize the pharmacological effects, or molecules that facilitate their tropism and retention in damaged tissues or recognition by target cells thus improving treatment selectivity. In addition, different approaches e.g. integration in a hydrogel-scaffold or chondroitin

sulfate sponge are in development to facilitate stable long-term delivery to joint tissues [174].

On the other hand, the determination of EVs can be useful as biomarkers of joint diseases as the content of microparticles is related to the parent cell and its microenvironment. In this respect, miRNA and proteome analyses represent promising approaches.

The standardization of isolation and characterization methods is crucial for the development of this novel tool. It is apparent that much work both *in vitro* and *in vivo* is needed in order to better understand the biogenesis, composition, appropriate delivery technique, *in vivo* stability and distribution, internalization, mechanisms of action, efficacy, long-term actions and safety of EVs.

Although we only focus on limited aspects of EVs, there are new mechanisms to be identified which may lead to other potential applications of these microparticles. Taken as a whole, the studies outlined in this review reinforce the increasing interest in the field and the efforts devoted to understand EV biology. Nevertheless, the complexity of the topic has raised a number of important questions which need to be answered before this novel approach can progress to clinical applications in joint conditions.

Acknowledgements: This work has been funded by grants SAF2013-48724R (MINECO, FEDER) and PROMETEOII/2014/071 (Generalitat Valenciana).

Conflict of interests: there is no conflict of interest to declare.

References

[1] Lener T, Gimona M, Aigner L, Borger V, Buzas E, Camussi G, Chaput N, Chatterjee D, Court FA, Del Portillo HA, O'Driscoll L, Fais S, Falcon-Perez JM, Felderhoff-Mueser U, Fraile L, Gho YS, Gorgens A, Gupta RC, Hendrix A, Hermann DM, Hill AF, Hochberg F, Horn PA, de KD, Kordelas L, Kramer BW, Kramer-Albers EM, Laner-Plamberger S, Laitinen S, Leonardi T, Lorenowicz MJ, Lim SK, Lotvall J, Maguire CA, Marcilla A, Nazarenko I, Ochiya T, Patel T, Pedersen S, Pocsfalvi G, Pluchino S, Quesenberry P, Reischl IG, Rivera FJ, Sanzenbacher R, Schallmoser K, Slaper-Cortenbach I, Strunk D, Tonn T, Vader P, van Balkom BW, Wauben M,

Andaloussi SE, Thery C, Rohde E, Giebel B. Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper. J Extracell Vesicles 2015;4:30087.

[2] Raposo G and Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 2013;200:373-83.

[3] Kalra H, Simpson RJ, Ji H, Aikawa E, Altevogt P, Askenase P, Bond VC, Borrás FE, Breakefield X, Budnik V. Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. PLoS Biol 2012;10.

[4] Simons M and Raposo G. Exosomes-vesicular carriers for intercellular communication. Curr Opin Cell Biol 2009;21:575-81.

[5] Bobrie A, Colombo M, Raposo G, Théry C. Exosome secretion: molecular mechanisms and roles in immune responses. Traffic 2011;12:1659-68.

[6] Firestein GS. Evolving concepts of rheumatoid arthritis. Nature 2003;423:356-61.

[7] Agca R, Heslinga SC, Rollefstad S, Heslinga M, McInnes IB, Peters MJ, Kvien TK, Dougados M, Radner H, Atzeni F, Primdahl J, Sodergren A, Wallberg JS, van RJ, Zabalan C, Pedersen TR, Jacobsson L, de VK, Gonzalez-Gay MA, Semb AG, Kitas GD, Smulders YM, Szekanecz Z, Sattar N, Symmons DP, Nurmohamed MT. EULAR recommendations for cardiovascular disease risk management in patients with rheumatoid arthritis and other forms of inflammatory joint disorders: 2015/2016 update. Ann Rheum Dis 2017;76:17-28.

[8] Reilly PA, Cosh JA, Maddison PJ, Rasker JJ, Silman AJ. Mortality and survival in rheumatoid arthritis: a 25 year prospective study of 100 patients. Ann Rheum Dis 1990;49:363-9.

[9] McInnes IB and Schett G. Pathogenetic insights from the treatment of rheumatoid arthritis. The Lancet 2017;389:2328-37.

[10] Goldring MB and Otero M. Inflammation in osteoarthritis. Curr Opin Rheumatol 2011;23:471-8.

[11] Abramson SB and Attur M. Developments in the scientific understanding of osteoarthritis. Arthritis Res Ther 2009;11:227.

[12] Sluijter JPG, Davidson SM, Boulanger CM, Buzas EI, de Kleijn DPV, Engel FB, Giricz Z, Hausenloy DJ, Kishore R, Lecour S, Leor J, Madonna R, Perrino C, Prunier F, Sahoo S, Schiffelers RM, Schulz R, Van Laake LW, Ytrehus K, Ferdinandy P. Extracellular vesicles in diagnostics and therapy of the ischaemic heart: Position Paper from the Working Group on

Cellular Biology of the Heart of the European Society of Cardiology. Cardiovasc Res 2018;114:19-34.

[13] Lacroix R, Judicone C, Mooberry M, Boucekine M, Key NS, Dignat-George F. Standardization of pre-analytical variables in plasma microparticle determination: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. J Thromb Haemost 2013;11:1190-3.

[14] Mitchell AJ, Gray WD, Hayek SS, Ko YA, Thomas S, Rooney K, Awad M, Roback JD, Quyyumi A, Searles CD. Platelets confound the measurement of extracellular miRNA in archived plasma. Sci Rep 2016;6:32651.

[15] Shelke GV, Lasser C, Gho YS, Lotvall J. Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum. J Extracell Vesicles 2014;3:24783.

[16] Taylor DD and Shah S. Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes. Methods 2015;87:3-10.

[17] Willms E, Johansson HJ, Mager I, Lee Y, Blomberg KE, Sadik M, Alaarg A, Smith CI, Lehtio J, El AS, Wood MJ, Vader P. Cells release subpopulations of exosomes with distinct molecular and biological properties. Sci Rep 2016;6:22519.

[18] Coumans FAW, Brisson AR, Buzas EI, Dignat-George F, Drees EEE, El-Andaloussi S, Emanueli C, Gasecka A, Hendrix A, Hill AF, Lacroix R, Lee Y, van Leeuwen TG, Mackman N, Mager I, Nolan JP, van der Pol E, Pegtel DM, Sahoo S, Siljander PRM, Sturk G, De WO, Nieuwland R. Methodological Guidelines to Study Extracellular Vesicles. Circ Res 2017;120:1632-48.

[19] Vogel R, Coumans FA, Maltesen RG, Boing AN, Bonnington KE, Broekman ML, Broom MF, Buzas EI, Christiansen G, Hajji N, Kristensen SR, Kuehn MJ, Lund SM, Maas SL, Nieuwland R, Osteikoetxea X, Schnoor R, Scicluna BJ, Shambrook M, de VJ, Mann SI, Hill AF, Pedersen S. A standardized method to determine the concentration of extracellular vesicles using tunable resistive pulse sensing. J Extracell Vesicles 2016;5:31242.

[20] Osteikoetxea X, Balogh A, Szabo-Taylor K, Nemeth A, Szabo TG, Paloczi K, Sodar B, Kittel A, Gyorgy B, Pallinger E, Matko J, Buzas EI. Improved characterization of EV preparations based on protein to lipid ratio and lipid properties. PLoS One 2015;10:e0121184.

[21] Gyorgy B, Szabo TG, Turiak L, Wright M, Herczeg P, Ledeczi Z, Kittel A, Polgar A, Toth K, Derfalvi B, Zelenak G, Borocz I, Carr B, Nagy G, Vekey K, Gay S, Falus A, Buzas EI.

Improved flow cytometric assessment reveals distinct microvesicle (cell-derived microparticle) signatures in joint diseases. PLoS One 2012;7:e49726.

[22] Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, Dingli F, Loew D, Tkach M, Théry C. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. PNAS 2016;113:E968-E977.

[23] van Pham P. Concise review: Extracellular vesicles from mesenchymal stem cells as cellular therapy, Biomed Res Ther 2017;4:1562-73.

[24] Durcin M, Fleury A, Taillebois E, Hilairet G, Krupova Z, Henry C, Truchet S, Trötzmüller M, Köfeler H, Mabilleau G, Hue O, Andriantsitohaina R, Martin P, Le Lay S. Characterisation of adipocyte-derived extracellular vesicle subtypes identifies distinct protein and lipid signatures for large and small extracellular vesicles. J Extracell Vesicles 2017;6:1305677.

[25] Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. J Proteomics 2010;73:1907-20.

[26] Eldh M, Ekstrom K, Valadi H, Sjostrand M, Olsson B, Jernas M, Lotvall J. Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA. PLoS ONE 2010;5:e15353.

[27] Kato T, Miyaki S, Ishitobi H, Nakamura Y, Nakasa T, Lotz MK, Ochi M. Exosomes from IL-1beta stimulated synovial fibroblasts induce osteoarthritic changes in articular chondrocytes. Arthritis Res Ther 2014;16:R163.

[28] Iraci N, Leonardi T, Gessler F, Vega B, Pluchino S. Focus on Extracellular Vesicles: Physiological Role and Signalling Properties of Extracellular Membrane Vesicles. Int J Mol Sci 2016;17:171.

[29] Kupcova Skalnikova H. Proteomic techniques for characterisation of mesenchymal stem cell secretome. Biochimie 2013;95:2196-211.

[30] Carpintero-Fernandez P, Fafian-Labora J, O'Loghlen A. Technical Advances to Study Extracellular Vesicles. Front Mol Biosci 2017;4:79.

[31] Record M, Carayon K, Poirot M, Silvente-Poirot S. Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies. Biochim Biophys Acta 2013;1841:108-20.

[32] Norling LV and Dalli J. Microparticles are novel effectors of immunity. Curr Opin Pharmacol 2013;13:570-5.

[33] Huang X, Yuan T, Tschannen M, Sun Z, Jacob H, Du M, Liang M, Dittmar RL, Liu Y, Liang M, Kohli M, Thibodeau SN, Boardman L, Wang L. Characterization of human plasma-derived exosomal RNAs by deep sequencing. BMC Genomics 2013;14:319.

[34] Kreimer S, Belov AM, Ghiran I, Murthy SK, Frank DA, Ivanov AR. Mass-spectrometry-based molecular characterization of extracellular vesicles: lipidomics and proteomics. J Proteome Res 2015;14:2367-84.

[35] Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9:654-9.

[36] Preusser C, Hung LH, Schneider T, Schreiner S, Hardt M, Moebus A, Santoso S, Bindereif A. Selective release of circRNAs in platelet-derived extracellular vesicles. J Extracell Vesicles 2018;7:1424473.

[37] Keerthikumar S, Chisanga D, Ariyaratne D, Al SH, Anand S, Zhao K, Samuel M, Pathan M, Jois M, Chilamkurti N, Gangoda L, Mathivanan S. ExoCarta: A Web-Based Compendium of Exosomal Cargo. J Mol Biol 2016;428:688-92.

[38] Kim, D.K., Lee, J., Kim, S.R., Choi, D.S., Yoon, Y.J., Kim, J.H., Go, G., Nhung, D., Hong, K., Jang, S.C., Kim, S.H., Park, K.S., Kim, O.Y., Park, H.T., Seo, J.H., Aikawa, E., Baj-Krzyworzeka, M., van Balkom, B.W., Belting, M., Blanc, L., Bond, V., Bongiovanni, A., Borras, F.E., Buee, L., Buzas, E.I., Cheng, L., Clayton, A., Cocucci, E., Dela Cruz, C.S., Desiderio, D.M., Di, Vizio D., Ekstrom, K., Falcon-Perez, J.M., Gardiner, C., Giebel, B., Greening, D.W., Gross, J.C., Gupta, D., Hendrix, A., Hill, A.F., Hill, M.M., Nolte-'t, Hoen E., Hwang, do W., Inal, J., Jagannadham, M.V., Jayachandran, M., Jee, Y.K., Jorgensen, M., Kim, K.P., Kim, Y.K., Kislinger, T., Lasser, C., Lee, D.S., Lee, H., van, Leeuwen J., Lener, T., Liu, M.L., Lotvall, J., Marcilla, A., Mathivanan, S., Moller, A., Morhayim, J., Mullier, F., Nazarenko, I., Nieuwland, R., Nunes, D.N., Pang, K., Park, J., Patel, T., Pocsfalvi, G., Del, Portillo H., Putz, U., Ramirez, M.I., Rodrigues, M.L., Roh, T.Y., Royo, F., Sahoo, S., Schiffelers, R., Sharma, S., Siljander, P., Simpson, R.J., Soekmadji, C., Stahl, P., Stensballe, A., Stepien, E., Tahara, H., Trummer, A., Valadi, H., Vella, L.J., Wai, S.N., Witwer, K., Yanez-Mo, M., Youn, H., Zeidler, R., Gho, Y.S. EVpedia: a community web portal for extracellular vesicles research: Bioinformatics 2015; 31, 933-939.

[39] Pathan M, Keerthikumar S, Chisanga D, Alessandro R, Ang CS, Askenase P, Batagov AO, Benito-Martin A, Camussi G, Clayton A, Collino F, Di VD, Falcon-Perez JM, Fonseca P, Fonseka P, Fontana S, Gho YS, Hendrix A, Hoen EN, Iraci N, Kastaniegaard K, Kislinger T, Kowal J, Kurochkin IV, Leonardi T, Liang Y, Llorente A, Lunavat TR, Maji S, Monteleone F,

Overbye A, Panaretakis T, Patel T, Peinado H, Pluchino S, Principe S, Ronquist G, Royo F, Sahoo S, Spinelli C, Stensballe A, Thery C, van Herwijnen MJC, Wauben M, Welton JL, Zhao K, Mathivanan S. A novel community driven software for functional enrichment analysis of extracellular vesicles data. J Extracell Vesicles 2017;6:1321455.

[40] Robbins PD and Morelli AE. Regulation of immune responses by extracellular vesicles. Nat Rev Immunol 2014;14:195-208.

[41] Robbins PD, Dorronsoro A, Booker CN. Regulation of chronic inflammatory and immune processes by extracellular vesicles. J Clin Invest 2016;126:1173-80.

[42] Tang K, Liu J, Yang Z, Zhang B, Zhang H, Huang C, Ma J, Shen GX, Ye D, Huang B. Microparticles mediate enzyme transfer from platelets to mast cells: A new pathway for lipoxin A4 biosynthesis. Biochemical and Biophysical Research Communications 2010;400:432-6.

[43] Njock MS, Cheng HS, Dang LT, Nazari-Jahantigh M, Lau AC, Boudreau E, Roufaiel M, Cybulsky MI, Schober A, Fish JE. Endothelial cells suppress monocyte activation through secretion of extracellular vesicles containing antiinflammatory microRNAs. Blood 2015;125:3202-12.

[44] Yang C and Robbins PD. Immunosuppressive exosomes: a new approach for treating arthritis. Int J Rheumatol 2012;2012:573528.

[45] Marton N, Kovacs OT, Baricza E, Kittel A, Gyori D, Mocsai A, Meier FMP, Goodyear CS, McInnes IB, Buzas EI, Nagy G. Extracellular vesicles regulate the human osteoclastogenesis: divergent roles in discrete inflammatory arthropathies. Cell Mol Life Sci 2017;74:3599-3611.

[46] Gelderman KA, Hultqvist M, Holmberg J, Olofsson P, Holmdahl R. T cell surface redox levels determine T cell reactivity and arthritis susceptibility. Proc Natl Acad Sci USA 2006;103:12831-6.

[47] Szabo-Taylor KE, Toth EA, Balogh AM, Sodar BW, Kadar L, Paloczi K, Fekete N, Nemeth A, Osteikoetxea X, Vukman KV, Holub M, Pallinger E, Nagy G, Winyard PG, Buzas EI. Monocyte activation drives preservation of membrane thiols by promoting release of oxidised membrane moieties via extracellular vesicles. Free Radic Biol Med 2017;108:56-65.

[48] Viegas CSB, Costa RM, Santos L, Videira PA, Silva Z, Araujo N, Macedo AL, Matos AP, Vermeer C, Simes DC. Gla-rich protein function as an anti-inflammatory agent in monocytes/macrophages: Implications for calcification-related chronic inflammatory diseases. PLoS One 2017;12:e0177829.

[49] Headland, S. E., and L. V. Norlin., The resolution of inflammation: Principles and challenges. Sem Immunol 2015; 27:149-160.

[50] Headland SE, Jones HR, Norling LV, Kim A, Souza PR, Corsiero E, Gil CD, Nerviani A, Dell'Accio F, Pitzalis C, Oliani SM, Jan LY, Perretti M. Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis. Sci Transl Med 2015;7:315ra190.

[51] Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan ML, Karlsson JM, Baty CJ, Gibson GA, Erdos G, Wang Z, Milosevic J, Tkacheva OA, Divito SJ, Jordan R, Lyons-Weiler J, Watkins SC, Morelli AE. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 2012;119:756-66.

[52] Kim SH, Lechman ER, Bianco N, Menon R, Keravala A, Nash J, Mi Z, Watkins SC, Gambotto A, Robbins PD. Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis. J Immunol 2005;174:6440-8.

[53] Bianco NR, Kim SH, Ruffner MA, Robbins PD. Therapeutic effect of exosomes from indoleamine 2,3-dioxygenase-positive dendritic cells in collagen-induced arthritis and delayed-type hypersensitivity disease models. Arthritis Rheum 2009;60:380-9.

[54] Kim SH, Bianco NR, Shufesky WJ, Morelli AE, Robbins PD. Effective treatment of inflammatory disease models with exosomes derived from dendritic cells genetically modified to express IL-4. J Immunol 2007;179:2242-9.

[55] Kim SH, Bianco N, Menon R, Lechman ER, Shufesky WJ, Morelli AE, Robbins PD. Exosomes derived from genetically modified DC expressing FasL are anti-inflammatory and immunosuppressive. Mol Ther 2006;13:289-300.

[56] Okoye IS, Coomes SM, Pelly VS, Czieso S, Papayannopoulos V, Tolmachova T, Seabra MC, Wilson MS. MicroRNA-containing T-regulatory-cell-derived exosomes suppress pathogenic T helper 1 cells. Immunity 2014;41:89-103.

[57] Aiello S, Rocchetta F, Longaretti L, Faravelli S, Todeschini M, Cassis L, Pezzuto F, Tomasoni S, Azzollini N, Mister M, Mele C, Conti S, Breno M, Remuzzi G, Noris M, Benigni A. Extracellular vesicles derived from T regulatory cells suppress T cell proliferation and prolong allograft survival. Sci Rep 2017;7:11518.

[58] Liu H and Pope RM. The role of apoptosis in rheumatoid arthritis. Curr Opin Pharmacol 2003;3:317-22.

[59] Martinez-Lorenzo MJ, Anel A, Gamen S, Monleon I, Lasierra P, Larrad L, Pineiro A, Alava MA, Naval J. Activated human T cells release bioactive Fas ligand and APO2 ligand in microvesicles. J Immunol 1999;163:1274-81.

[60] Martinez-Lorenzo MJ, Anel A, Saez-Gutierrez B, Royo-Canas M, Bosque A, Alava MA, Pineiro A, Lasierra P, Asin-Ungria J, Larrad L. Rheumatoid synovial fluid T cells are sensitive to APO2L/TRAIL. Clin Immunol 2007;122:28-40.

[61] Martinez-Lostao L, Garcia-Alvarez F, Basanez G, Alegre-Aguaron E, Desportes P, Larrad L, Naval J, Martinez-Lorenzo MJ, Anel A. Liposome-bound APO2L/TRAIL is an effective treatment in a rabbit model of rheumatoid arthritis. Arthritis Rheum 2010;62:2272-82.

[62] Arntz OJ, Pieters BC, Oliveira MC, Broeren MG, Bennink MB, de VM, van Lent PL, Koenders MI, van den Berg WB, van der Kraan PM, van de Loo FA. Oral administration of bovine milk derived extracellular vesicles attenuates arthritis in two mouse models. Mol Nutr Food Res 2015;59:1701-12.

[63] Pistoia V and Raffaghello L. Mesenchymal stromal cells and autoimmunity. Int Immunol 2017;29:49-58.

[64] Quaedackers ME, Baan CC, Weimar W, Hoogduijn MJ. Cell contact interaction between adipose-derived stromal cells and allo-activated T lymphocytes. Eur J Immunol 2009;39:3436-46.

[65] Ren G, Zhao X, Zhang L, Zhang J, L'Huillier A, Ling W, Roberts AI, Le AD, Shi S, Shao C, Shi Y. Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. J Immunol 2010;184:2321-8.

[66] Meisel R, Zibert A, Laryea M, G+Âbel U, D+ñubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood 2004;103:4619-21.

[67] Aggarwal S and Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815-22.

[68] Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2008;2:141-50.

[69] Chabannes D, Hill M, Merieau E, Rossignol J, Brion R, Soulillou JP, Anegon I, Cuturi MC. A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells. Blood 2007;110:3691-4.

[70] Bernardo ME and Fibbe WE. Mesenchymal Stromal Cells: Sensors and Switchers of Inflammation. Cell Stem Cell 2013;13:392-402.

[71] Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, Borg C, Saas P, Tiberghien P, Rouas-Freiss N, Carosella ED, Deschaseaux F. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. Stem Cells 2008;26:212-22.

[72] Di Nicola M., Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 2002;99:3838-43.

[73] Hofer HR and Tuan RS. Secreted trophic factors of mesenchymal stem cells support neurovascular and musculoskeletal therapies. Stem Cell Res Ther 2016;7:131.

[74] Matula Z, Nemeth A, Lorincz P, Szepesi A, Brozik A, Buzas EI, Low P, Nemet K, Uher F, Urban VS. The Role of Extracellular Vesicle and Tunneling Nanotube-Mediated Intercellular Cross-Talk Between Mesenchymal Stem Cells and Human Peripheral T Cells. Stem Cells Dev 2016;25:1818-32.

[75] Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, Noiseux N, Zhang L, Pratt RE, Ingwall JS. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 2005;11:367-8.

[76] Tang YL, Zhao Q, Qin X, Shen L, Cheng L, Ge J, Phillips MI. Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. Ann Thorac Surg 2005;80:229-36.

[77] Togel F, Hu Z, Weiss K, Isaac J, Lange C, Westenfelder C. Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. Am J Physiol Renal Physiol 2005;289:F31-F42.

[78] Togel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C. Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. Am J Physiol Renal Physiol 2007;292:F1626-F1635.

[79] Lai RC, Chen TS, Lim SK. Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. Regen Med 2011;6:481-92.

[80] Timmers L, Lim SK, Arslan F, Armstrong JS, Hoefer IE, Doevendans PA, Piek JJ, El Oakley RM, Choo A, Lee CN, Pasterkamp G, de Kleijn DP. Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. Stem Cell Res 2007;1:129-37.

[81] Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, Oakley RM. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. Stem Cell Res 2010;4:214-22.

[82] Bruno S, Grange C, Deregibus MC, Calogero RA, Saviozzi S, Collino F, Morando L, Busca A, Falda M, Bussolati B, Tetta C, Camussi G. Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. J Am Soc Nephrol 2009;20:1053-67.

[83] Szabo GT, Tarr B, Paloczi K, Eder K, Lajko E, Kittel A, Toth S, Gyorgy B, Pasztoi M, Nemeth A, Osteikoetxea X, Pallinger E, Falus A, Szabo-Taylor K, Buzas EI. Critical role of extracellular vesicles in modulating the cellular effects of cytokines. Cell Mol Life Sci 2014;71:4055-67.

[84] Kordelas L, Rebmann V, Ludwig AK, Radtke S, Ruesing J, Doeppner TR, Epple M, Horn PA, Beelen DW, Giebel B. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. Leukemia 2014;28:970-3.

[85] Nassar W, El-Ansary M, Sabry D, Mostafa MA, Fayad T, Kotb E, Temraz M, Saad AN, Essa W, Adel H. Umbilical cord mesenchymal stem cells derived extracellular vesicles can safely ameliorate the progression of chronic kidney diseases. Biomater Res 2016;20:21.

[86] Zhou BR, Xu Y, Guo SL, Xu Y, Wang Y, Zhu F, Permatasari F, Wu D, Yin ZQ, Luo D. The effect of conditioned media of adipose-derived stem cells on wound healing after ablative fractional carbon dioxide laser resurfacing. Biomed Res Int 2013;2013:519126.

[87] Fukuoka H and Suga H. Hair Regeneration Treatment Using Adipose-Derived Stem Cell Conditioned Medium: Follow-up With Trichograms. Eplasty 2015;15:e10.

[88] Raik S, Kumar A, Bhattacharyya S. Insights into cell-free therapeutic approach: Role of stem cell "soup-ernatant". Biotechnol Appl Biochem 2017 [Epub ahead of print].

[89] Di TM, Bassi G, Midolo M, Gatti A, Kamga PT, Cassaro A, Carusone R, Adamo A, Krampera M. Differential and transferable modulatory effects of mesenchymal stromal cell-derived extracellular vesicles on T, B and NK cell functions. Sci Rep 2016;6:24120.

[90] Budoni M, Fierabracci A, Luciano R, Petrini S, Di C, V, Muraca M. The immunosuppressive effect of mesenchymal stromal cells on B lymphocytes is mediated by membrane vesicles. Cell Transplant 2013;22:369-79.

[91] Fierabracci A, Del FA, Luciano R, Muraca M, Teti A, Muraca M. Recent advances in mesenchymal stem cell immunomodulation: the role of microvesicles. Cell Transplant 2015;24:133-49.

[92] Blazquez R, Sanchez-Margallo FM, de la Rosa O, Dalemans W, Alvarez V, Tarazona R, Casado JG. Immunomodulatory Potential of Human Adipose Mesenchymal Stem Cells Derived Exosomes on in vitro Stimulated T Cells. Front Immunol 2014;5:556.

[93] Conforti A, Scarsella M, Starc N, Giorda E, Biagini S, Proia A, Carsetti R, Locatelli F, Bernardo ME. Microvescicles derived from mesenchymal stromal cells are not as effective as their cellular counterpart in the ability to modulate immune responses in vitro. Stem Cells Dev 2014;23:2591-9.

[94] Del FA, Luciano R, Pascucci L, Goffredo BM, Giorda E, Scapaticci M, Fierabracci A, Muraca M. Immunoregulatory Effects of Mesenchymal Stem Cell-Derived Extracellular Vesicles on T Lymphocytes. Cell Transplant 2015;24:2615-27.

[95] Harting MT, Srivastava AK, Zhaorigetu S, Bair H, Prabhakara KS, Toledano Furman NE, Vykoukal JV, Ruppert KA, Cox CS, Jr., Olson SD. Inflammation-Stimulated Mesenchymal Stromal Cell-Derived Extracellular Vesicles Attenuate Inflammation. Stem Cells 2018;36:79-90.

[96] Cosenza S, Toupet K, Luz-Crawford P, Jorgensen C, Noël D. Mesenchymal stem cell-derived exosomes and microvesicles exert similar immunomodulatory effect in collagen-induced arthritis. Ann Rheum Dis 2017;76:A61.

[97] Lo Sicco C, Reverberi D, Balbi C, Ulivi V, Principi E, Pascucci L, Becherini P, Bosco MC, Varesio L, Franzin C, Pozzobon M, Cancedda R, Tasso R. Mesenchymal stem cell-derived extracellular vesicles as mediators of anti-Inflammatory effects: Endorsement of macrophage polarization. Stem Cells Transl Med 2017;6:1018-28.

[98] Henao Agudelo JS, Braga TT, Amano MT, Cenedeze MA, Cavinato RA, Peixoto-Santos AR, Muscará MN, Teixeira SA, Cruz MC, Castoldi A, Sinigaglia-Coimbra R, Pacheco-Silva A, de Almeida DC, Camara NOS. Mesenchymal stromal cell-derived microvesicles regulate an internal pro-Inflammatory program in activated macrophages. Frontiers in Immunology 2017;8:881.

[99] Zhang B, Yin Y, Lai RC, Tan SS, Choo AB, Lim SK. Mesenchymal stem cells secrete immunologically active exosomes. Stem Cells Dev 2014;23:1233-44.

[100] Monguio-Tortajada M, Roura S, Galvez-Monton C, Pujal JM, Aran G, Sanjurjo L, Franquesa M, Sarrias MR, Bayes-Genis A, Borras FE. Nanosized UCMSC-derived extracellular vesicles but not conditioned medium exclusively inhibit the inflammatory response of stimulated T cells: implications for nanomedicine. Theranostics 2017;7:270-84.

[101] Casado JG, Blazquez R, Vela FJ, Alvarez V, Tarazona R, Sanchez-Margallo FM. Mesenchymal stem cell-derived exosomes: Immunomodulatory evaluation in an antigen-induced synovitis porcine model. Front Vet Sci 2017;4:39.

[102] Lai RC, Tan SS, Teh BJ, Sze SK, Arslan F, de Kleijn DP, Choo A, Lim SK. Proteolytic potential of the MSC exosome proteome: Implications for an exosome-mediated delivery of therapeutic proteasome. Int J Proteomics 2012;2012:971907.

[103] Sanjabi S, Oh SA, Li MO. Regulation of the immune response by TGF-beta: From conception to autoimmunity and infection. Cold Spring Harb Perspect Biol 2017;9:a022236.

[104] Tian G, Li JL, Wang DG, Zhou D. Targeting IL-10 in auto-immune diseases. Cell Biochem Biophys 2014;70:37-49.

[105] Mokarizadeh A, Delirezh N, Morshedi A, Mosayebi G, Farshid AA, Mardani K. Microvesicles derived from mesenchymal stem cells: potent organelles for induction of tolerogenic signaling. Immunol Lett 2012;147:47-54.

[106] Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, Sharpe AH. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. J Exp Med 2009;206:3015-29.

[107] Bianco GA, Toscano MA, Ilarregui JM, Rabinovich GA. Impact of protein-glycan interactions in the regulation of autoimmunity and chronic inflammation. Autoimmun Rev 2006;5:349-56.

[108] Garin MI, Chu CC, Golshayan D, Cernuda-Morollon E, Wait R, Lechler RI. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. Blood 2007;109:2058-65.

[109] Rabinovich GA, Daly G, Dreja H, Tailor H, Riera CM, Hirabayashi J, Chernajovsky Y. Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. J Exp Med 1999;190:385-98.

[110] Collino F, Deregibus MC, Bruno S, Sterpone L, Aghemo G, Viltono L. Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stem cells shuttle selected pattern of miRNAs. PLoS One 2010;5:e11803.

[111] Vicente R, Noel D, Pers YM, Apparailly F, Jorgensen C. Deregulation and therapeutic potential of microRNAs in arthritic diseases. Nat Rev Rheumatol 2016;12:211-20.

[112] Counsel PD, Bates D, Boyd R, Connell DA. Cell therapy in joint disorders. Sports Health 2015;7:27-37.

[113] Rojas M, Xu J, Woods CR, Mora AL, Spears W, Roman J, Brigham KL. Bone marrow-derived mesenchymal stem cells in repair of the injured lung. Am J Respir Cell Mol Biol 2005;33:145-52.

[114] Farrell MJ, Comeau ES, Mauck RL. Mesenchymal stem cells produce functional cartilage matrix in three-dimensional culture in regions of optimal nutrient supply. Eur Cell Mater 2012;23:425-40.

[115] Itokazu M, Wakitani S, Mera H, Tamamura Y, Sato Y, Takagi M, Nakamura H. Transplantation of Scaffold-Free Cartilage-Like Cell-Sheets Made from Human Bone Marrow Mesenchymal Stem Cells for Cartilage Repair: A Preclinical Study. Cartilage 2016;7:361-72.

[116] Xie X, Wang Y, Zhao C, Guo S, Liu S, Jia W, Tuan RS, Zhang C. Comparative evaluation of MSCs from bone marrow and adipose tissue seeded in PRP-derived scaffold for cartilage regeneration. Biomaterials 2012;33:7008-18.

[117] Nejadnik H, Hui JH, Feng Choong EP, Tai BC, Lee EH. Autologous bone marrow-derived mesenchymal stem cells versus autologous chondrocyte implantation: an observational cohort study. Am J Sports Med 2010;38:1110-6.

[118] Sekiya I, Muneta T, Horie M, Koga H. Arthroscopic Transplantation of Synovial Stem Cells Improves Clinical Outcomes in Knees With Cartilage Defects. Clin Orthop Relat Res 2015;473:2316-26.

[119] Kim YS, Choi YJ, Lee SW, Kwon OR, Suh DS, Heo DB, Koh YG. Assessment of clinical and MRI outcomes after mesenchymal stem cell implantation in patients with knee osteoarthritis: a prospective study. Osteoarthritis Cartilage 2016;24:237-45.

[120] de Windt TS, Vonk LA, Slaper-Cortenbach IC, van den Broek MP, Nizak R, van Rijen MH, de Weger RA, Dhert WJ, Saris DB. Allogeneic mesenchymal stem cells stimulate cartilage regeneration and are safe for single-stage cartilage repair in humans upon mixture with recycled autologous chondrons. Stem Cells 2017;35:256-64.

[121] Kim YS, Kwon OR, Choi YJ, Suh DS, Heo DB, Koh YG. Comparative matched-pair analysis of the injection versus implantation of mesenchymal stem cells for knee osteoarthritis. Am J Sports Med 2015:43:2738-46.

[122] Davatchi F, Sadeghi AB, Mohyeddin M, Nikbin B. Mesenchymal stem cell therapy for knee osteoarthritis: 5 years follow-up of three patients. Int J Rheum Dis 2016;19:219-25.

[123] Orozco L, Munar A, Soler R, Alberca M, Soler F, Huguet M, Sentis J, Sanchez A, Garcia-Sancho J. Treatment of Knee Osteoarthritis With Autologous Mesenchymal Stem Cells: A Pilot Study. Transplantation 2013;95:1535-41.

[124] Lee WY and Wang B. Cartilage repair by mesenchymal stem cells: Clinical trial update and perspectives. J Orthop Translat 2017;9:76-88.

[125] Wong KL, Lee KBL, Tai BC, Law P, Lee EH, Hui JHP. Injectable cultured bone marrow-derived mesenchymal stem cells in varus knees with cartilage defects undergoing high tibial osteotomy: A prospective, randomized controlled clinical trial with 2 years' follow-up. Arthroscopy 2013;29:2020-8.

[126] Park YB, Ha CW, Lee CH, Yoon YC, Park YG. Cartilage oegeneration in Osteoarthritic patients by a composite of allogeneic umbilical cord blood-derived mesenchymal stem cells and hyaluronate Hydrogel: Results from a clinical trial for safety and proof-of-concept with 7 years of extended follow-up. Stem Cells Transl Med 2017;6:613-21.

[127] Murphy MB, Moncivais K, Caplan AI. Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. Exp Mol Med 2013;45:e54.

[128] Gomez-Aristizabal A, Sharma A, Bakooshli MA, Kapoor M, Gilbert PM, Viswanathan S, Gandhi R. Stage-specific differences in secretory profile of mesenchymal stromal cells (MSCs) subjected to early- vs late-stage OA synovial fluid. Osteoarthritis Cartilage 2017;25:737-41.

[129] Tomchuck SL, Zwezdaryk KJ, Coffelt SB, Waterman RS, Danka ES, Scandurro AB. Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. Stem Cells 2008;26:99-107.

[130] Ti D, Hao H, Tong C, Liu J, Dong L, Zheng J, Zhao Y, Liu H, Fu X, Han W. LPS-preconditioned mesenchymal stromal cells modify macrophage polarization for resolution of chronic inflammation via exosome-shuttled let-7b. J Transl Med 2015;13:308.

[131] Osugi M, Katagiri W, Yoshimi R, Inukai T, Hibi H, Ueda M. Conditioned media from mesenchymal stem cells enhanced bone regeneration in rat calvarial bone defects. Tissue Eng Part A 2012;18:1479-89.

[132] Kawai T, Katagiri W, Osugi M, Sugimura Y, Hibi H, Ueda M. Secretomes from bone marrow-derived mesenchymal stromal cells enhance periodontal tissue regeneration. Cytotherapy 2015;17:369-81.

[133] Ogata K, Katagiri W, Osugi M, Kawai T, Sugimura Y, Hibi H, Nakamura S, Ueda M. Evaluation of the therapeutic effects of conditioned media from mesenchymal stem cells in a rat bisphosphonate-related osteonecrosis of the jaw-like model. Bone 2015;74:95-105.

[134] Katagiri W, Osugi M, Kawai T, Hibi H. First-in-human study and clinical case reports of the alveolar bone regeneration with the secretome from human mesenchymal stem cells. Head Face Med 2016;12:5.

[135] Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. Annu Rev Cell Dev Biol 2007;23:435-61.

[136] Arasu UT, Karna R, Harkonen K, Oikari S, Koistinen A, Kroger H, Qu C, Lammi MJ, Rilla K. Human mesenchymal stem cells secrete hyaluronan-coated extracellular vesicles. Matrix Biol 2017;64:54-68.

[137] de Jong OG, van Balkom BW, Schiffelers RM, Bouten CV, Verhaar MC. Extracellular vesicles: potential roles in regenerative medicine. Front Immunol 2014;5:608.

[138] de Jong OG, Verhaar MC, Chen Y, Vader P, Gremmels H, Posthuma G, Schiffelers RM, Gucek M, van Balkom BW. Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes. J Extracell Vesicles 2012;1:18396.

[139] Athens AA, Makris EA, Hu JC. Induced collagen cross-links enhance cartilage integration. PLoS ONE 2013;8:e60719.

[140] Borges FT, Melo SA, Ozdemir BC, Kato N, Revuelta I, Miller CA, Gattone VH, LeBleu VS, Kalluri R. TGF-beta1-containing exosomes from injured epithelial cells activate fibroblasts to initiate tissue regenerative responses and fibrosis. J Am Soc Nephrol 2013;24:385-92.

[141] Tomasoni S, Longaretti L, Rota C, Morigi M, Conti S, Gotti E, Capelli C, Introna M, Remuzzi G, Benigni A. Transfer of growth factor receptor mRNA via exosomes unravels the regenerative effect of mesenchymal stem cells. Stem Cells Dev 2013;22:772-80.

[142] Liu X, Yang Y, Li Y, Niu X, Zhao B, Wang Y, Bao C, Xie Z, Lin Q, Zhu L. Integration of stem cell-derived exosomes with in situ hydrogel glue as a promising tissue patch for articular cartilage regeneration. Nanoscale 2017;9:4430-8.

[143] Vonk LA, van Dooremalen S, Liv N, Klumperman J, Coffer P, Saris D, Lorenowicz M. Mesenchymal Stromal/stem Cell-derived Extracellular Vesicles Promote Human Cartilage Regeneration In Vitro. Theranostics 2018;8:906-20.

[144] Cosenza S, Ruiz M, Toupet K, Jorgensen C, Noel D. Mesenchymal stem cells derived exosomes and microparticles protect cartilage and bone from degradation in osteoarthritis. Sci Rep 2017;7:16214.

[145] Tofiño-Vian M, Guillén MI, Pérez del Caz MD, Castejón MA, Alcaraz MJ. Extracellular Vesicles from Adipose-Derived Mesenchymal Stem Cells Downregulate Senescence Features in Osteoarthritic Osteoblasts. Oxid Med Cell Longev 2017;2017:12 pages.

[146] Cosenza S, Ruiz M, Maumus M, Jorgensen C, Noel D. Pathogenic or therapeutic extracellular vesicles in rheumatic diseases: Role of mesenchymal stem cell-derived vesicles. Int J Mol Sci 2017;18:889.

[147] Nakamura Y, Miyaki S, Ishitobi H, Matsuyama S, Nakasa T, Kamei N. Mesenchymal-stem-cell-derived exosomes accelerate skeletal muscle regeneration. FEBS Lett 2015;589:1257-65.

[148] Qin Y, Wang L, Gao Z, Chen G, Zhang C. Bone marrow stromal/stem cell-derived extracellular vesicles regulate osteoblast activity and differentiation in vitro and promote bone regeneration in vivo. Sci Rep 2016;6:21961.

[149] Liu X, Li Q, Niu X, Hu B, Chen S, Song W, Ding J, Zhang C, Wang Y. Exosomes secreted from human-induced pluripotent stem cell-derived mesenchymal stem cells prevent osteonecrosis of the femoral head by promoting angiogenesis. Int J Biol Sci 2017;13:232-44.

[150] Qi X, Zhang J, Yuan H, Xu Z, Li Q, Niu X, Hu B, Wang Y, Li X. Exosomes secreted by human-induced pluripotent stem cell-derived mesenchymal stem cells repair critical-sized bone defects through enhanced angiogenesis and osteogenesis in osteoporotic rats. Int J Biol Sci 2016;12:836-49.

[151] Zhang J, Liu X, Li H, Chen C, Hu B, Niu X, Li Q, Zhao B, Xie Z, Wang Y. Exosomes/tricalcium phosphate combination scaffolds can enhance bone regeneration by activating the PI3K/Akt signaling pathway. Stem Cell ResTher 2016;7:136.

[152] Zhang S, Chu WC, Lai RC, Lim SK, Hui JH, Toh WS. Exosomes derived from human embryonic mesenchymal stem cells promote osteochondral regeneration. Osteoarthritis Cartilage 2016;24:2135-40.

[153] Tao SC, Yuan T, Zhang YL, Yin WJ, Guo SC, Zhang CQ. Exosomes derived from miR-140-5p-overexpressing human synovial mesenchymal stem cells enhance cartilage tissue regeneration and prevent osteoarthritis of the knee in a rat model. Theranostics 2017;7:180-95.

[154] Zhu Y, Wang Y, Zhao B, Niu X, Hu B, Li Q, Zhang J, Ding J, Chen Y, Wang Y. Comparison of exosomes secreted by induced pluripotent stem cell-derived mesenchymal stem cells and synovial membrane-derived mesenchymal stem cells for the treatment of osteoarthritis. Stem Cell Res Ther 2017;8:64.

[155] Rey-Rico A, Reinecke J, Wehling P, Cucchiarini M, Madry H. Effects of exosomes upon the metabolic activities of human osteoarthritic articular cartilage in situ. Osteoarthritis Cartilage 2015;23:A399.

[156] Ponsaerts P and Berneman ZN. Modulation of cellular behavior by exogenous messenger RNA. Leukemia 2006;20:767-9.

[157] Quesenberry PJ, Dooner MS, Aliotta JM. Stem cell plasticity revisited: the continuum marrow model and phenotypic changes mediated by microvesicles. Exp Hematol 2010;3838:581-92.

[158] Greene MA and Loeser RF. Aging-related inflammation in osteoarthritis. Osteoarthritis and Cartilage 2015;23:1966-71.

[159] Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. Arthritis Rheum 2012;64:1697-707.

[160] Toh WS, Lai RC, Hui JH, Lim SK. MSC exosome as a cell-free MSC therapy for cartilage regeneration: Implications for osteoarthritis treatment. Semin Cell Dev Biol 2016.

[161] Fahy N, de Vries-van Melle ML, Lehmann J, Wei W, Grotenhuis N, Farrell E, van der Kraan PM, Murphy JM, Bastiaansen-Jenniskens YM, van Osch GJ. Human osteoarthritic synovium impacts chondrogenic differentiation of mesenchymal stem cells via macrophage polarisation state. Osteoarthritis Cartilage 2014;22:1167-75.

[162] Ding J, Chen B, Lv T, Liu X, Fu X, Wang Q, Yan L, Kang N, Cao Y, Xiao R. Bone Marrow Mesenchymal Stem Cell-Based Engineered Cartilage Ameliorates Polyglycolic Acid/Polylactic Acid Scaffold-Induced Inflammation Through M2 Polarization of Macrophages in a Pig Model. Stem Cells Transl Med 2016;5:1079-89.

[163] Chen TS, Lai RC, Lee MM, Choo AB, Lee CN, Lim SK. Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. Nucleic Acids Res 2010;38:215-24.

[164] Ham O, Song BW, Lee SY, Choi E, Cha MJ, Lee CY, Park JH, Kim IK, Chang W, Lim S, Lee CH, Kim S, Jang Y, Hwang KC. The role of microRNA-23b in the differentiation of MSC into chondrocyte by targeting protein kinase A signaling. Biomaterials 2012;33:4500-7.

[165] Kim D, Song J, Jin EJ. MicroRNA-221 regulates chondrogenic differentiation through promoting proteosomal degradation of slug by targeting Mdm2. J Biol Chem 2010;285:26900-7.

[166] Ning G, Liu X, Dai M, Meng A, Wang Q. MicroRNA-92a upholds Bmp signaling by targeting noggin3 during pharyngeal cartilage formation. Dev Cell 2013;24:283-95.

[167] Hou C, Zhang Z, Zhang Z, Wu P, Zhao X, Fu M, Sheng P, Kang Y, Liao W. Presence and function of microRNA-92a in chondrogenic ATDC5 and adipose-derived mesenchymal stem cells. Mol Med Rep 2015;12:4877-86.

[168] Matsukawa T, Sakai T, Yonezawa T, Hiraiwa H, Hamada T, Nakashima M, Ono Y, Ishizuka S, Nakahara H, Lotz MK, Asahara H, Ishiguro N. MicroRNA-125b regulates the

expression of aggrecanase-1 (ADAMTS-4) in human osteoarthritic chondrocytes. Arthritis Research & Therapy 2013;15:R28.

[169] Meng F, Zhang Z, Chen W, Huang G, He A, Hou C, Long Y, Yang Z, Zhang Z, Liao W. MicroRNA-320 regulates matrix metalloproteinase-13 expression in chondrogenesis and interleukin-1beta-induced chondrocyte responses. Osteoarthritis Cartilage 2016;24:932-41.

[170] Zhang R, Ma J, Yao J. Molecular mechanisms of the cartilage-specific microRNA-140 in osteoarthritis. Inflamm Res 2013;62:871-7.

[171] Distler JH, Pisetsky DS, Huber LC, Kalden JR, Gay S, Distler O. Microparticles as regulators of inflammation: novel players of cellular crosstalk in the rheumatic diseases. Arthritis Rheum 2005;52:3337-48.

[172] Beyer C and Pisetsky DS. The role of microparticles in the pathogenesis of rheumatic diseases. Nat Rev Rheumatol 2010;6:21-9.

[173] Dye JR, Ullal AJ, Pisetsky DS. The role of microparticles in the pathogenesis of rheumatoid arthritis and systemic lupus erythematosus. Scand J Immunol 2013;78:140-8.

[174] Withrow J, Murphy C, Liu Y, Hunter M, Fulzele S, Hamrick MW. Extracellular vesicles in the pathogenesis of rheumatoid arthritis and osteoarthritis. Arthritis Res Ther 2016;18:286.

[175] Foers AD, Cheng L, Hill AF, Wicks IP, Pang KC. Review: Extracellular Vesicles in Joint Inflammation. Arthritis Rheumatol 2017;69:1350-62.

[176] Fu H, Hu D, Zhang L, Tang P. Role of extracellular vesicles in rheumatoid arthritis. Mol Immunol 2018;93:125-32.

[177] Bardelli C, Amoruso A, Federici CD, Fresu L, Balbo P, Neri T, Celi A, Brunelleschi S. Autocrine activation of human monocyte/macrophages by monocyte-derived microparticles and modulation by PPARgamma ligands. Br J Pharmacol 2012;165:716-28.

[178] André F, Chaput N, Schartz NEC, Flament C, Aubert N, Bernard J, Lemonnier F, Raposo G, Escudier B, Hsu DH, Tursz T, Amigorena S, Angevin E, Zitvogel L. Exosomes as Potent Cell-Free Peptide-Based Vaccine. I. Dendritic Cell-Derived Exosomes Transfer Functional MHC Class I/Peptide Complexes to Dendritic Cells. J Immunol 2004;172:2126.

[179] Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ. B lymphocytes secrete antigen-presenting vesicles. J Exp Med 1996;183.

[180] Pasztoi M, Sodar B, Misjak P, Paloczi K, Kittel A, Toth K, Wellinger K, Geher P, Nagy G, Lakatos T, Falus A, Buzas EI. The recently identified hexosaminidase D enzyme substantially

contributes to the elevated hexosaminidase activity in rheumatoid arthritis. Immunol Lett 2013;149:71-6.

[181] Lo CA, Majkowska I, Nagase H, Di L, I, Troeberg L. Microvesicles shed by oligodendroglioma cells and rheumatoid synovial fibroblasts contain aggrecanase activity. Matrix Biol 2012;31:229-33.

[182] Rosenthal AK. Articular cartilage vesicles and calcium crystal deposition diseases. Curr Opin Rheumatol 2016;28:127-32.

[183] Rosenthal AK, Gohr CM, Mitton-Fitzgerald E, Grewal R, Ninomiya J, Coyne CB, Jackson WT. Autophagy modulates articular cartilage vesicle formation in primary articular chondrocytes. J Biol Chem 2015;290:13028-38.

[184] He C, Zheng S, Luo Y, Wang B. Exosome Theranostics: Biology and Translational Medicine. Theranostics 2018;8:237-55.

[185] Mahendran SM, Oikonomopoulou K, Diamandis EP, Chandran V. Synovial fluid proteomics in the pursuit of arthritis mediators: An evolving field of novel biomarker discovery. Crit Rev Clin Lab Sci 2017;54:495-505.

[186] Pant S, Hilton H, Burczynski ME. The multifaceted exosome: biogenesis, role in normal and aberrant cellular function, and frontiers for pharmacological and biomarker opportunities. Biochem Pharmacol 2012;83:1484-94.

[187] Rodriguez-Carrio J, Alperi-Lopez M, Lopez P, Alonso-Castro S, Carro-Esteban SR, Ballina-Garcia FJ, Suarez A. Altered profile of circulating microparticles in rheumatoid arthritis patients. Clin Sci (Lond) 2015;128:437-48.

[188] Knijff-Dutmer EAJ, Koerts J, Nieuwland R, Kalsbeek-Batenburg EM, Van De Laar MAFJ. Elevated levels of platelet microparticles are associated with disease activity in rheumatoid arthritis. Arthritis Rheum 2002; 46:1498-503

[189] van Eijk IC, Tushuizen ME, Sturk A, Dijkmans BAC, Boers M, Voskuyl AE, Diamant M, Wolbink GJ, Nieuwland R, Nurmohamed MT. Circulating microparticles remain associated with complement activation despite intensive anti-inflammatory therapy in early rheumatoid arthritis. Ann Rheum Dis 2010;69:1378.

[190] Yoo J, Lee SK, Lim M, Sheen D, Choi EH, Kim SA. Exosomal amyloid A and lymphatic vessel endothelial hyaluronic acid receptor-1 proteins are associated with disease activity in rheumatoid arthritis. Arthritis Res Ther 2017;19:119.

[191] Song J, Kim D, Han J, Kim Y, Lee M, Jin EJ. PBMC and exosome-derived Hotair is a critical regulator and potent marker for rheumatoid arthritis. Clin Exp Med 2015;15:121-6.

[192] Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, Massarotti EM, Remold-O'Donnell E, Farndale RW, Ware J, Lee DM. Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. Science 2010;327:580-3.

[193] Skriner K, Adolph K, Jungblut PR, Burmester GR. Association of citrullinated proteins with synovial exosomes. Arthritis Rheum 2006;54:3809-14.

[194] Fan W, Wang W, Wu J, Ma L, Guo J. Identification of CD4(+) T-cell-derived CD161(+) CD39(+) and CD39(+)CD73(+) microparticles as new biomarkers for rheumatoid arthritis. Biomark Med 2017;11:107-16.

[195] Murphy C, Withrow J, Hunter M, Liu Y, Tang YL, Fulzele S, Hamrick MW. Emerging role of extracellular vesicles in musculoskeletal diseases. Mol Aspects Med 2017 (in press).

[196] Kolhe R, Hunter M, Liu S, Jadeja RN, Pundkar C, Mondal AK, Mendhe B, Drewry M, Rojiani MV, Liu Y, Isales CM, Guldberg RE, Hamrick MW, Fulzele S. Gender-specific differential expression of exosomal miRNA in synovial fluid of patients with osteoarthritis. Sci Rep 2017;7:2029.

[197] van der Pol E, Boing AN, Gool EL, Nieuwland R. Recent developments in the nomenclature, presence, isolation, detection and clinical impact of extracellular vesicles. J Thromb Haemost 2016;14:48-56.

[198] Maas SL, Breakefield XO, Weaver AM. Extracellular Vesicles: Unique Intercellular Delivery Vehicles. Trends Cell Biol 2017;27:172-88.

[199] Reiner AT, Witwer KW, van Balkom BWM, de BJ, Brodie C, Corteling RL, Gabrielsson S, Gimona M, Ibrahim AG, de KD, Lai CP, Lotvall J, Del Portillo HA, Reischl IG, Riazifar M, Salomon C, Tahara H, Toh WS, Wauben MHM, Yang VK, Yang Y, Yeo RWY, Yin H, Giebel B, Rohde E, Lim SK. Concise Review: Developing Best-Practice Models for the Therapeutic Use of Extracellular Vesicles. Stem Cells Transl Med 2017;6:1730-9.

[200] Van DJ, Mestdagh P, Sormunen R, Cocquyt V, Vermaelen K, Vandesompele J, Bracke M, De WO, Hendrix A. The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling. J Extracell Vesicles 2014;3.

[201] Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol 2011;13:423-33.

[202] Van DJ, Mestdagh P, Agostinis P, Akay O, Anand S, Anckaert J, Martinez ZA, Baetens T, Beghein E, Bertier L, Berx G, Boere J, Boukouris S, Bremer M, Buschmann D, Byrd JB, Casert C, Cheng L, Cmoch A, Daveloose D, De SE, Demirsoy S, Depoorter V, Dhondt B, Driedonks TA, Dudek A, Elsharawy A, Floris I, Foers AD, Gartner K, Garg AD, Geeurickx E, Gettemans J, Ghazavi F, Giebel B, Kormelink TG, Hancock G, Helsmoortel H, Hill AF, Hyenne V, Kalra H, Kim D, Kowal J, Kraemer S, Leidinger P, Leonelli C, Liang Y, Lippens L, Liu S, Lo CA, Martin S, Mathivanan S, Mathiyalagan P, Matusek T, Milani G, Monguio-Tortajada M, Mus LM, Muth DC, Nemeth A, Nolte-'t Hoen EN, O'Driscoll L, Palmulli R, Pfaffl MW, Primdal-Bengtson B, Romano E, Rousseau Q, Sahoo S, Sampaio N, Samuel M, Scicluna B, Soen B, Steels A, Swinnen JV, Takatalo M, Thaminy S, Thery C, Tulkens J, Van A, I, van der Grein S, Van GA, van Herwijnen MJ, Van NG, Van RN, Van Vliet AR, Vandamme N, Vanhauwaert S, Vergauwen G, Verweij F, Wallaert A, Wauben M, Witwer KW, Zonneveld MI, De WO, Vandesompele J, Hendrix A. EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research. Nat Methods 2017;14:228-32.

[203] Vozel D, Ursic B, Krek JL, Stukelj R, Kralj-Iglic V. Applicability of extracellular vesicles in clinical studies. Eur J Clin Invest 2017;47:305-13.

[204] Maguire G. Stem cell therapy without the cells. Commun Integr Biol 2013;6:e26631.

[205] Rasmusson I, Le BK, Sundberg B, Ringden O. Mesenchymal stem cells stimulate antibody secretion in human B cells. Scand J Immunol 2007;65:336-43.

[206] Biancone L, Bruno S, Deregibus MC, Tetta C, Camussi G. Therapeutic potential of mesenchymal stem cell-derived microvesicles. Nephrol Dial Transplant 2012;27:3037-42.

[207] Cha JM, Shin EK, Sung JH, Moon GJ, Kim EH, Cho YH, Park HD, Bae H, Kim J, Bang OY. Efficient scalable production of therapeutic microvesicles derived from human mesenchymal stem cells. Sci Rep 2018;8:1171.

DISCUSIÓN

1. Caracterización de las vesículas extracelulares

Todos los fluidos corporales contienen vesículas celulares de membrana. Tales EVs son liberadas por células procariotas y eucariotas con mensajes que transportan a su entorno. Se cree que estas vesículas contribuyen a la homeostasis, el desarrollo y progresión de enfermedades por lo que podrían proporcionar nuevos biomarcadores y nuevas estrategias terapéuticas (Coumans, Brisson et al. 2017).

Sin embargo, varias controversias y dificultades metodológicas han obstaculizado el conocimiento de estas EVs y sus funciones biológicas. En primer lugar, su descubrimiento simultáneo en distintas áreas del conocimiento ha propiciado la generación de una nomenclatura dispersa y confusa según criterios dispares de propiedades físicas, biogénesis y función (Gould y Raposo 2013). En esta tesis doctoral hemos adoptado la nomenclatura más comúnmente aceptada, que define MVs y EXs como partículas de distinto tamaño medio y mecanismo de biogénesis diferencial. No obstante, parece que será necesaria a medio plazo la aplicación de una terminología basada exclusivamente en tamaño, con el apoyo de marcadores más o menos específicos identificados en subpoblaciones vesiculares.

Los marcadores habitualmente definidos como universales son altamente variables según el origen celular y entorno fisiológico de las EVs, y no parece probable que sea posible, al menos en un futuro próximo, identificar EVs experimentalmente según su biogénesis (Kowal, Arras et al. 2016). Dada la heterogeneidad e impureza de las preparaciones de EVs, muchos de los efectos biológicos atribuidos a éstas podrían deberse a la presencia de componentes no vesiculares (Carpintero-Fernández, Fafián-Labora et al. 2017). La necesidad de estandarizar la metodología de aislamiento y caracterización ha sido reconocida por la comunidad científica y recogida por ISEV, la cual ha publicado distintas guías y recomendaciones para la adecuada definición y caracterización de las EVs en los protocolos experimentales (Coumans, Brisson et al. 2017).

Recientemente se ha propuesto el uso de EVs con fines terapéuticos para reemplazar las terapias celulares, más complejas, más difíciles de controlar y cuyo resultado tras la implantación puede ser impredecible (Lasser, Jang et al. 2018). Por ejemplo, el uso específico de células madre como las ASCs conlleva el riesgo adicional de oncogénesis (Raik, Kumar et al. 2018).

En este trabajo se han aislado las EVs provenientes de ASCs por centrifugación acoplada a filtración diferencial y se han caracterizado en términos de tamaño medio, morfología general y composición proteica mediante distintas técnicas de análisis como TRPS, microscopía electrónica y espectrometría de masas. La combinación de TRPS y microscopía electrónica permitió identificar las dos poblaciones de vesículas con la morfología específica descrita en la literatura y el rango de tamaños referido a las MVs (en torno a 100-600 nm) y los EXs (30-150 nm).

El análisis de espectrometría de masas permitió identificar proteínas específicas de las fracciones de MVs y EXs en comparación con el CM de ASCs. El bajo número de proteínas enriquecidas en EXs en comparación con el CM (sólo 3 con un incremento de al menos 2,5 puntos) puede deberse a la presencia de contaminantes séricos co-precipitados en la ultracentrifugación. Como se ha comentado anteriormente, muchos de estos contaminantes no desaparecen tras el lavado de las muestras (Coumans, Brisson et al. 2017). El mismo fenómeno puede explicar el menor número de proteínas no compartidas entre EXs y CM. Así, resulta problemático el estudio proteico de productos derivados de células cultivadas en presencia de porcentajes elevados de suero, cuyos componentes son, por otra parte, imprescindibles para el metabolismo celular. Aunque se han desarrollado sustitutivos del suero humano en forma de sueros sintéticos o mezclas enriquecidas y diluidas en ciertos factores, este refleja más fielmente el entorno fisiológico celular, lo que tiene relevancia cuando se estudia la función biológica.

No obstante, algunas proteínas identificadas en las EVs podrían desempeñar un papel regulador en nuestras condiciones experimentales. En particular, hemos identificado anexina A1, una proteína con conocidos efectos antiinflamatorios y resolutivos, en las MVs de ASCs (Cloix, Colard et al. 1983, Six y Dennis 2000, Ferlazzo, D'Agostino et al. 2003). Otras proteínas identificadas podrían tener un papel relevante en los efectos específicos de las MVs. Por ejemplo, hemos observado la sobreexpresión de peroxirredoxina 6, integrante de la familia de proteínas antioxidantes tiol-específicas, cuya actividad glutatión peroxidasa contribuye a controlar el estrés oxidativo a través de la reducción de H_2O_2, ácidos grasos de cadena corta e hidroperóxidos de fosfolípidos (Fisher 2017). En el contexto de la fisiología ósea, se sabe que la acumulación de lípidos oxidados activa la señalización osteoblástica del receptor de peroxisoma-proliferador-activado γ, inhibiendo la formación de hueso y activando la adipogénesis de las MSCs (Kassem y Marie 2011).

Además de anexina A1 y peroxirredoxina 6, detectamos varias proteínas relacionadas con el citosqueleto relevantes en el contexto del cartílago articular: la integrina β1, la tetraspanina CD81, las tropomiosinas 1 y 3, la α-actinina 4 y el homólogo 3 de la familia fermitina. Estas proteínas cumplen distintas funciones en el complejo transmembrana de señalización de integrinas, anclando la célula a determinados ligandos de la matriz extracelular y activando rutas de señalización a través de las proteínas adaptadoras del citoesqueleto (Fletcher y Mullins 2010). En concreto, los ligandos de integrina β1 son específicamente colágeno de tipo II y fibronectina y la señalización derivada de la interacción contribuye a mantener el fenotipo fisiológico de los condrocitos articulares. En OA, una expresión anormal de integrina β1 altera la señalización célula-matriz y modifica la expresión génica en condrocitos y osteoblastos, provocando un desequilibrio a favor de proteínas catabólicas y mediadores pro-inflamatorios (Tian, Zhang et al. 2015).

Las tres proteínas significativamente sobreexpresadas en EXs derivados de ASCs fueron la subunidad 2 de carboxipeptidasa N (CPN), la proteína de zona de gestación (PZP) y la chaperona Hsc70. La primera proteína ha sido relativamente poco estudiada y se corresponde con la subunidad reguladora de CPN. CPN es una zinc-metaloproteasa de proteínas inflamatorias, especialmente de proteínas del complemento como C3a o C5a, bradiquinina o creatina quinasa. Por otra parte, dado que CPN corta específicamente lisinas y argininas carboxi-terminales, y la arginina es uno de los principales sustratos para la producción de NO por iNOS, se ha propuesto que CPN podría inhibir la producción de NO en focos inflamatorios locales (Matthews, Mueller-Ortiz et al. 2004).

Por su parte, PZP recibe su nombre por constituir uno de los componentes principales del suero en las últimas etapas de la gestación. Es capaz de inhibir las cuatro clases de proteinasas mediante un mecanismo de "captura" a través de una cola peptídica que actúa de cebo. Cuando una proteinasa escinde este cebo, PZP experimenta un cambio conformacional que envuelve a la proteinasa y se une covalentemente a ella, inactivándola. Por tanto, es considerada un potente inhibidor de proteasas (Wyatt, Cater et al. 2016).

Por último, Hsc70 es una chaperona molecular y uno de los marcadores exosomales más conocidos. Participa en procesos celulares diversos, principalmente relacionados con el plegado y la degradación de proteínas (Liu, Daniels et al. 2012). Además, es uno de los principales responsables de la autofagia mediada por chaperonas, un proceso crítico para el recambio homeostático de proteínas y estructuras disfuncionales (Cuervo y Wong

2014). Existen evidencias de que la autofagia disminuye con el envejecimiento y especialmente durante la progresión de la OA. En este sentido, se ha propuesto que la autofagia podría tener un efecto protector en estadios avanzados de la enfermedad (Benderdour, Martel-Pelletier et al. 2015).

2. Actividad biológica de las vesículas extracelulares

Una vez estudiadas las características y composición proteica, abordamos la efectividad de las MVs y EXs para controlar las diversas alteraciones observadas en los condrocitos y osteoblastos osteoartríticos, que mantienen una estrecha relación con la inflamación, la pérdida del fenotipo fisiológico y la adopción de características catabólicas y senescentes. En este sentido, nuestro grupo ha demostrado previamente las propiedades antiinflamatorias (Platas, Guillén et al. 2013) y antisenescentes (Platas, Guillén et al. 2016) del CM proveniente de ASCs en condrocitos osteoartríticos estimulados con IL-1β. En general, las evidencias indican que la efectividad de las terapias celulares con MSCs, que promueven la regeneración tisular en distintas enfermedades, es consecuencia de la señalización paracrina que estas células dirigen (Ratajczak, Jadczyk et al. 2014).

En la OA, la inflamación es crítica tanto para el establecimiento como para la progresión y agravamiento de la enfermedad. Los condrocitos y sinoviocitos participan en un círculo de retroalimentación positiva en el espacio sinovial, intercambiando mensajes que agravan la activación inflamatoria de ambos tipos celulares, y que activa la síntesis de enzimas degradativas y mediadores proinflamatorios. Como consecuencia, se produce la degradación de la matriz extracelular, cuya integridad es crítica para el funcionamiento fisiológico del cartílago articular, y el reclutamiento de células inflamatorias, empeorando inexorablemente la funcionalidad de la articulación (Goldring y Otero 2011).

En primer lugar, hemos analizado comparativamente distintos sistemas de aislamiento de EVs, encontrando que la centrifugación diferencial sigue siendo un método válido para el análisis de subpoblaciones de EVs a partir de elevados de volúmenes de CM. Asimismo, hemos comprobado en ensayos preliminares que tanto las MVs como los EXs producen un efecto dependiente de la dosis (los estudios preliminares para la elección y refinamiento del método de aislamiento, así como el hallazgo del efecto dosis-dependiente se recogen en el anexo I). Además, las concentraciones utilizadas durante los estudios no son tóxicas para las células.

En segundo lugar, nuestro trabajo ha demostrado que las EVs producidas por ASCs son las responsables principales del efecto paracrino antiinflamatorio de dichas células. La presencia de IL-1β en la articulación osteoartrítica induce la expresión de un amplio rango de mediadores proinflamatorios, desde citocinas y quimiocinas hasta PGE_2, NO y enzimas degradativas como MMPs (Goldring y Otero 2011). Las MVs y EXs presentes en el CM proveniente del cultivo de ASCs humanas ejercen efectos antiinflamatorios que son específicos de estas vesículas, al menos en condrocitos osteoartríticos. En comparación, la aplicación de EVs provenientes de un origen distinto y no relacionado (una línea celular de queratinocitos adultos) no replica estos efectos. Es decir, la actividad antiinflamatoria de las EVs es propia de aquellas derivadas de ASCs, y no de componentes ubicuos en todas las preparaciones.

Las EVs provenientes de ASCs demostraron efectos antiinflamatorios tanto en condrocitos como en osteoblastos osteoartríticos estimulados con IL-1β. En ambos tipos celulares, el tratamiento con las EVs inhibió la liberación de IL-6 y PGE_2. IL-6 es una citocina proinflamatoria de gran relevancia en el contexto osteoartrítico. Está involucrada en la fisiopatología de la enfermedad (Goekoop, Kloppenburg et al. 2010), controlando las alteraciones en el metabolismo del condrocito de forma tanto autocrina como paracrina, a través de osteoblastos que liberan citocinas a la placa ósea subcondral y el cartílago calcificado que inducen mecanismos de señalización en los condrocitos (Findlay y Atkins 2014). Esta sobreexpresión de IL-6 en la articulación osteoartrítica promueve la degradación de la matriz extracelular tanto en hueso como en cartílago, contribuyendo decisivamente en la progresión de la enfermedad. De hecho, IL-6 es el marcador inflamatorio más estrechamente asociado con la edad y la fragilidad en la enfermedad (Greene y Loeser 2015).

Por su parte, la sobreproducción de PGE_2 puede inhibir la formación de hueso y mediar la osteoclastogénesis inducida por IL-6 (Tai, Miyaura et al. 1997), retroalimentando además la producción de IL-6 por parte de los osteoblastos (Massicotte, Lajeunesse et al. 2002). En condrocitos, PGE_2 induce cambios catabólicos y degradativos que agravan la destrucción articular (Li, Afif et al. 2005). Nuestros resultados indican que las EVs son capaces de controlar los niveles de este eicosanoide tanto en condrocitos como en osteoblastos, lo que podría contrarrestar las consecuencias de la inflamación crónica en la articulación. En condrocitos, específicamente, observamos que las EVs regulan

negativamente la transcripción de COX-2 y mPGES-1, principales responsables en la síntesis de PGE_2.

Las EVs provenientes de ASCs también promovieron la producción de IL-10 en condrocitos y osteoblastos. Este mediador bloquea la síntesis de varias citocinas proinflamatorias, entre ellas IL-1β, así como la producción de ROS (Dokka, Shi et al. 2001) y PGE_2 (Alaaeddine, Di Battista et al. 1999). En condrocitos, el tratamiento de MVs indujo más la síntesis de IL-10 en comparación con EXs. Esto podría estar relacionado con su contenido en anexina A1. Esta proteína puede activar al receptor de péptido formilado (FPR)2, que fosforila a p38 tras su activación, lo que lleva a la fosforilación de la quinasa 2 de MAPK (MAPKAPK2) y la proteína de estrés térmico 27 y, por último, la producción de IL-10. Hasta el momento, esta ruta de activación se ha descrito únicamente en monocitos y macrófagos (Headland y Norling 2015).

En contraste, el tratamiento con EVs redujo significativamente la producción de TNFα en condrocitos pero no en osteoblastos. Esta citocina promueve de forma decisiva la liberación de mediadores proinflamatorios, contribuyendo a la retroalimentación positiva que degrada la articulación irreversiblemente. Sin embargo, se ha observado que TNFα también puede favorecer la diferenciación osteogénica a través de NF-κB, incrementando la expresión de la proteína morfogénica ósea 2, osterix, el factor de transcripción relacionado con Runt 2, osteocalcina y la ruta Wnt (Osta, Benedetti et al. 2014).

En condrocitos, además, las EVs fueron capaces de reducir la actividad MMP global e inhibieron la transcripción de MMP-13, cuya importancia es crítica en el contexto articular al ser una de las principales enzimas degradativas del colágeno de tipo II (Goldring, Otero et al. 2011). Esto se pudo correlacionar con la inhibición transcripcional de iNOS y una reducción en la liberación de NO, un mediador que contribuye en la activación de MMPs con la consiguiente degradación de la matriz extracelular, indispensable para el funcionamiento fisiológico del cartílago articular (Lotz 1999).

La señalización canónica de NF-κB en OA es responsable de muchos de los efectos inflamatorios descritos: este factor controla la inducción de mediadores inflamatorios, mecanismos catabólicos y cambios fenotípicos que favorecen el inicio y progresión de la enfermedad (Olivotto, Otero et al. 2015). Por otra parte, el factor AP-1 (proteína activadora 1) coopera con e incrementa los efectos de NF-κB al inducir la expresión de MMPs como MMP-13 (Liacini, Sylvester et al. 2002). En conjunto, estos dos factores

generan un fenotipo inflamatorio y catabólico que se retroalimenta por la presencia creciente de mediadores proinflamatorios. En este sentido, nuestro trabajo ha constatado que el tratamiento con EVs es capaz de inhibir la unión a DNA de ambos factores.

La presencia de anexina A1 en las MVs podría explicar parte de sus propiedades antiinflamatorias. Anexina A1, también conocida como renocortina, macrocortina o lipocortina-1, se identificó por primera vez como un factor capaz de inhibir la fosfolipasa A_2 citosólica ($cPLA_2$) (Cloix, Colard et al. 1983). Las propiedades proinflamatorias de $cPLA_2$ son bien conocidas: libera ácido araquidónico, mediador inflamatorio y precursor de otras moléculas como leucotrienos y prostaglandinas, incluyendo PGE_2 (Six y Dennis 2000). Además, se ha observado que anexina A1 controla la liberación de NO a través de la inhibición de iNOS y la sobreproducción de IL-10 en macrófagos (Ferlazzo, D'Agostino et al. 2003). Al mismo tiempo, inhibe la expresión de COX-2 en células de la microglia (Minghetti, Nicolini et al. 1999).

Por otra parte, la anexina A1 parece tener una relación con la activación de NF-κB, si bien las evidencias son por ahora contradictorias. En linfocitos T, por ejemplo, anexina A1 causa la activación de las rutas NF-κB y AP-1 (D'Acquisto, Merghani et al. 2007), mientras que la acción del péptido MC-12 de anexina A1 inhibe NF-κB en un modelo animal de colitis (Ouyang, Zhu et al. 2012, Sheikh y Solito 2018). Nuestros estudios proteómicos han identificado anexina A1 en las MVs pero no en los EXs, lo que podría estar relacionado con la mayor capacidad de inhibición de NF- κB por las MVs respecto a los EXs.

Se sabe que la expresión de colágeno de tipo II en la matriz extracelular es uno de los principales indicativos del fenotipo característico del condrocito articular. Las fibras de colágeno, a su vez, constituyen uno de los elementos estructurales más importantes del cartílago. Esta expresión se pierde conforme el proceso osteoartrítico progresa en la articulación (Martel-Pelletier, Barr et al. 2016), lo que desencadena cambios fenotípicos en la célula al perder la señalización dependiente de colágeno en la matriz e integrinas en la membrana plasmática (Tian, Zhang et al. 2015). *In vitro*, la estimulación con IL-1β inhibe la producción de colágeno de tipo II (Goldring y Otero 2011).

Por tanto, mantener el fenotipo condrocítico, medido según la expresión de colágeno de tipo II, es importante en el desarrollo de terapias para el tratamiento de la OA. Nuestros experimentos indican que las EVs producidas por ASCs protegen a los condrocitos

osteoartríticos del efecto supresor de IL-1β sobre la expresión de colágeno tipo II. Esta propiedad, unida a su capacidad para disminuir la actividad MMP —una de las principales responsables de la degradación crónica del colágeno articular—, determina el papel protector potencial de estas EVs en el tejido articular.

En el progreso de la OA se produce una senescencia debida al estrés celular por la presencia de una inflamación crónica de bajo grado. Esta inflamación provoca estrés oxidativo y alteraciones celulares que derivan en una senescencia independiente del acortamiento telomérico (Itahana, Campisi et al. 2004). El fenotipo secretor propio de las células senescentes agrava la condición inflamatoria por la producción de citocinas, factores de crecimiento y MMPs que degradan el tejido articular e inducen cambios fenotípicos catabólicos y apoptóticos (Tchkonia, Zhu et al. 2013). En el contexto osteoartrítico, la senescencia se ha explorado con mayor profundidad en los condrocitos, células altamente diferenciadas con poco recambio celular. En osteoblastos, la senescencia celular es la causa principal de pérdida de hueso relacionada con la edad tanto en hombres como en mujeres mayores de 50 años (Kassem y Marie 2011). La secreción de mediadores inflamatorios como IL-6 como parte del fenotipo senescente puede inducir cambios proinflamatorios en los condrocitos y sinoviocitos articulares.

Se ha observado que la presencia y liberación de ROS produce una disfunción de la actividad osteoblástica y la formación de hueso durante el envejecimiento, lo que viene asociado con una reducción de la actividad glutatión reductasa y una activación de $p66^{SHC}$, una proteína adaptadora que amplifica la generación de ROS mitocondrial y la señalización apoptótica (Manolagas 2010; Kassem y Marie 2011). De hecho, la mayor parte de las ROS son producidas por las mitocondrias como resultado de la fosforilación oxidativa, que genera un potencial de protones ($\Delta\Psi$) a través de la membrana mitocondrial interna. Estas ROS pueden dañar tanto los componentes mitocondriales como los citosólicos y nucleares. Además, inducen la oxidación descontrolada de proteínas, alterando su función (Balaban, Nemoto et al. 2005).

Por otra parte, la exposición del DNA a ROS puede provocar daño genómico. Si la maquinaria reparadora de la célula es incapaz de reparar a tiempo este daño, la acumulación de errores de secuencia produce mutaciones somáticas que generan proteínas disfuncionales y deletéreas, conduciendo potencialmente a la célula a procesos cancerígenos y/o apoptóticos. Distintos modelos animales han mostrado el impacto del

daño al DNA en la formación de hueso, principalmente por inactivación de p53 (Kassem y Marie 2011).

En este sentido, nuestros resultados indican que el tratamiento con MVs y EXs derivadas de ASCs es capaz de inhibir diversas características propias de la senescencia en osteoblastos osteoartríticos. En primer lugar, las EVs fueron capaces de disminuir la actividad β-galactosidasa asociada a senescencia tras la estimulación con IL-1β (Carnero 2013). Como se ha comentado anteriormente, el fenotipo secretor es de importancia crítica en el contexto de la OA ya que las células senescentes secretan factores pro-inflamatorios como IL-6, empeorando progresivamente la fisiología de la articulación (Greene y Loeser 2015). Por otra parte, las EVs fueron capaces de reducir la presencia de focos de γH2AX, una histona que se acumula en el núcleo tras detectar roturas en la doble cadena de DNA, así como de restaurar el potencial de membrana mitocondrial medido en las células no estimuladas con IL-1β.

En conjunto, nuestros resultados muestran que el tratamiento con EVs derivadas de ASCs reproduce los efectos antiinflamatorios y regenerativos del CM, y podría constituir una alternativa de terapia en el futuro. En ese sentido, sería relevante detallar si las EVs tienen efectos antisenescentes también en condrocitos, en los que la senescencia inducida por estrés contribuye a la pérdida de fenotipo y la secreción de mediadores inflamatorios (Greene y Loeser 2015). Asimismo, sería interesante comprobar si estas EVs tienen algún efecto a nivel de regulación de la autofagia, ya que contienen proteínas con el potencial para hacerlo, como Hsc70. Se sabe que la autofagia es un mecanismo protector de las células sometidas a estrés, y su disminución en OA es uno de los causantes de muerte celular en condrocitos y osteoblastos (Martel-Pelletier, Barr et al. 2016). Por otra parte, uno de los mecanismos que contribuye de manera decisiva en el mantenimiento y progreso de la OA es el continuo intercambio de moléculas entre los condrocitos articulares y los sinoviocitos, promoviendo mecanismos de retroalimentación que dificultan la restauración del fenotipo fisiológico. Explorar la comunicación entre estos dos tipos celulares tras el tratamiento con EVs aportaría información muy significativa sobre su utilidad terapéutica. Por último, ampliar nuestro conocimiento sobre las proteínas presentes en los EXs de ASCs permitiría esbozar nuevas hipótesis sobre los mecanismos de acción responsables de su efecto.

Hoy en día, la OA es una enfermedad que continúa careciendo de tratamiento regenerativo. Los fármacos de uso clínico están indicados para el alivio sintomático. Las

intervenciones quirúrgicas que reemplazan la articulación dañada con implantes no resuelven el estado inflamatorio crónico, lo que desemboca habitualmente en la degradación del implante y la necesidad de nuevas intervenciones, con un deterioro notable de la calidad de vida del paciente. Es, por otra parte, una patología con una elevada prevalencia a partir de los 50 años, implicando un elevado coste social y también una carga económica importante sobre los sistemas públicos sanitarios. El desarrollo de estrategias terapéuticas que mitiguen la inflamación crónica y promuevan la regeneración articular es, por tanto, de gran importancia, y el uso de EVs derivadas de ASCs puede contribuir de manera decisiva en este camino.

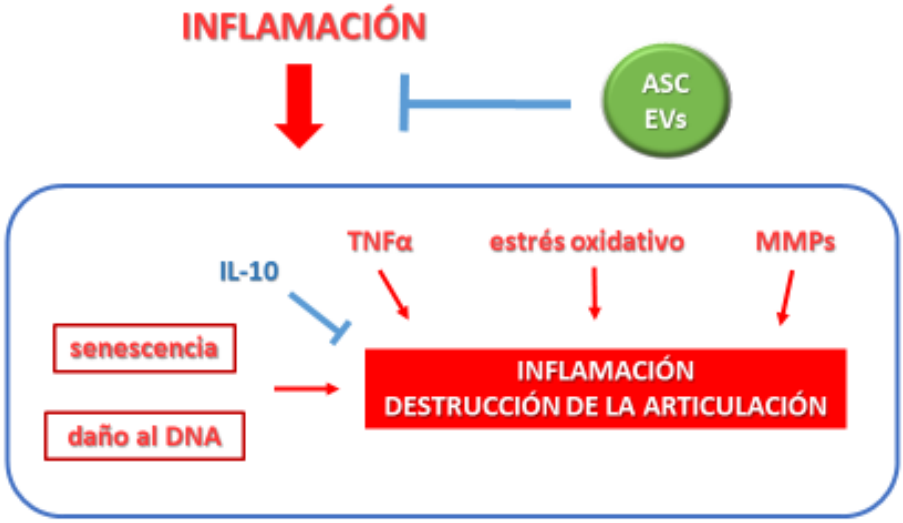

Figura 7. Resumen de los efectos protectores de las EVs derivadas de ASCs sobre la articulación. En azul se engloban los procesos inflamatorios, catabólicos, oxidativos y senescentes que las EVs inhiben o, en el caso de IL-10, promueven en la articulación osteoartrítica.

CONCLUSIONES / *CONCLUSIONS*

1. Se han aislado y caracterizado las vesículas extracelulares derivadas de células madre mesenquimales de tejido adiposo por técnicas de análisis de tamaño, como el TRPS; y morfología, como la microscopía electrónica. Asimismo, se ha estudiado su composición proteica por espectrometría de masas, encontrando tanto proteínas comunes a otras vesículas como algunas específicas tales como anexina A1, una proteína antiinflamatoria.

2. Las vesículas extracelulares reproducen el efecto antiinflamatorio del medio acondicionado, inhibiendo la liberación de IL-6 y PGE_2, mientras que promueven la expresión de IL-10 en condrocitos y osteoblastos osteoartríticos. En condrocitos osteoartríticos inhiben la producción de NO, TNFα, la actividad MMP global, la activación de los factores de transcripción NF-κB y AP-1 y la expresión génica de COX-2 y mPGES-1, MMP-13 e iNOS. Además, mantienen el fenotipo condrocítico en condiciones inflamatorias.

3. Las vesículas extracelulares tienen propiedades antioxidantes y antisenescentes en osteoblastos osteoartríticos, inhibiendo la actividad β-galactosidasa asociada a senescencia, la formación de focos γH2AX y la peroxidación lipídica, mientras que restauran el potencial de la membrana mitocondrial interna.

4. Nuestros resultados indican que las vesículas extracelulares derivadas de células madre mesenquimales de tejido adiposo son efectores relevantes del secretoma de dichas células y representan una estrategia potencial para el desarrollo de nuevas terapias en patologías inflamatorias crónicas articulares.

1. Adipose tissue-derived mesenchymal stem cell extracellular vesicles have been isolated and characterized by size analysis methods, like TRPS; and morphology analysis methods, such as electron microscopy. Also, their protein composition has been studied by mass spectrometry, finding common vesicle proteins as well as some specific, such as annexin A1, an anti-inflammatory protein.

2. These extracellular vesicles reproduce the anti-inflammatory effect of the conditioned medium, inhibiting the liberation of IL-6 and PGE_2, while promoting the expression of IL-10 in osteoarthritic chondrocytes and osteoblasts. In osteoarthritic chondrocytes, they inhibit the production of NO, TNFα, global MMP activity, transcription factors NF-κB and AP-1 activation, and the gene expression of COX-2, mPGES-1, MMP-13 and iNOS. Also, they contribute to maintain the chondrocyte phenotype in inflammatory conditions.

3. Extracellular vesicles have antioxidant and antisenescent effects in osteoarthritic osteoblasts, inhibiting senescence-associated β-galactosidase activity, formation of γH2AX foci and lipid peroxidation, while restoring the normal electric potential of the inner mitochondrial layer.

4. Our results indicate that the extracellular vesicles derived from adipose tissue mesenchymal stem cells are relevant secretome effectors, and they represent a potential strategy for the development of new therapies in joint chronic inflammatory pathologies.

BIBLIOGRAFÍA

Abbasi-Malati, Z., A. M. Roushandeh, Y. Kuwahara y M. H. Roudkenar (2018). "Mesenchymal stem cells on horizon: a new arsenal of therapeutic agents." Stem Cell Rev. **14**(4): 484-499.

Alaaeddine, N., J. A. Di Battista, J. P. Pelletier, K. Kiansa, J. M. Cloutier y J. Martel-Pelletier (1999). "Inhibition of tumor necrosis factor alpha-induced prostaglandin E2 production by the antiinflammatory cytokines interleukin-4, interleukin-10, and interleukin-13 in osteoarthritic synovial fibroblasts: distinct targeting in the signaling pathways." Arthritis Rheum **42**(4): 710-718.

Anderson, W., R. Lane, D. Korbie y M. Trau (2015). "Observations of tunable resistive pulse sensing for exosome analysis: improving system sensitivity and stability." Langmuir **31**(23): 6577-6587.

Armstrong, D. y D. E. Wildman (2018). "Extracellular vesicles and the promise of continuous liquid biopsies." J Pathol Transl Med **52**(1): 1-8.

Bacakova, L., J. Zarubova, M. Travnickova, J. Musilkova, J. Pajorova, P. Slepicka, N. S. Kasalkova, V. Svorcik, Z. Kolska, H. Motarjemi y M. Molitor (2018). "Stem cells: their source, potency and use in regenerative therapies with focus on adipose-derived stem cells - a review." Biotechnol Adv. **36**(4): 1111-1126.

Balaban, R. S., S. Nemoto y T. Finkel (2005). "Mitochondria, oxidants, and aging." Cell **120**(4): 483-495.

Benderdour, M., J. Martel-Pelletier, J. P. Pelletier, M. Kapoor, M. V. Zunzunegui y H. Fahmi (2015). "Cellular Aging, senescence and autophagy processes in osteoarthritis." Curr Aging Sci **8**(2): 147-157.

Bernardo, M. E. y W. E. Fibbe (2013). "Mesenchymal stromal cells: sensors and switchers of inflammation." Cell Stem Cell **13**(4): 392-402.

Boing, A. N., E. van der Pol, A. E. Grootemaat, F. A. Coumans, A. Sturk y R. Nieuwland (2014). "Single-step isolation of extracellular vesicles by size-exclusion chromatography." J Extracell Vesicles **3**. DOI: 10.3402/jev.v3.23430.

Bolukbasi, M. F., A. Mizrak, G. B. Ozdener, S. Madlener, T. Strobel, E. P. Erkan, J. B. Fan, X. O. Breakefield y O. Saydam (2012). "miR-1289 and "zipcode"-like sequence enrich mrnas in microvesicles." Mol Ther Nucleic Acids **1**: e10.

Buzás, E. I., C. Gardiner, C. Lee y Z. J. Smith (2017). "Single particle analysis: Methods for detection of platelet extracellular vesicles in suspension (excluding flow cytometry)." Platelets **28**(3): 249-255.

Carnero, A. (2013). "Markers of cellular senescence." Methods Mol Biol **965**: 63-81.

Carpintero-Fernández, P., J. Fafián-Labora y A. O'Loghlen (2017). "Technical advances to study extracellular vesicles." Frontiers in Molecular Biosciences **4**: 79.

Chargaff, E. y R. West (1946). "The biological significance of the thromboplastic protein of blood." J Biol Chem **166**(1): 189-197.

Clérigues, V., M. I. Guillén, F. Gomar y M. J. Alcaraz (2012). "Haem oxygenase-1 counteracts the effects of interleukin-1β on inflammatory and senescence markers in cartilage-subchondral bone explants from osteoarthritic patients." Clin Sci (Lond) **122**(5): 239-250.

Cloix, J. F., O. Colard, B. Rothhut y F. Russo-Marie (1983). "Characterization and partial purification of 'renocortins': two polypeptides formed in renal cells causing the anti-phospholipase-like action of glucocorticoids." Br J Pharmacol **79**(1): 313-321.

Colombo, M., G. Raposo y C. Théry (2014). "Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles." Annual Review of Cell and Developmental Biology **30**(1): 255-289.

Contreras, R. A., F. E. Figueroa, F. Djouad y P. Luz-Crawford (2016). "Mesenchymal stem cells regulate the innate and adaptive immune responses dampening arthritis progression." Stem Cells Int **2016**: 3162743.

Coumans, F. A., E. van der Pol, A. N. Boing, N. Hajji, G. Sturk, T. G. van Leeuwen y R. Nieuwland (2014). "Reproducible extracellular vesicle size and concentration determination with tunable resistive pulse sensing." J Extracell Vesicles **3**: 25922.

Coumans, F. A. W., A. R. Brisson, E. I. Buzas, F. Dignat-George, E. E. E. Drees, S. El-Andaloussi, C. Emanueli, A. Gasecka, A. Hendrix, A. F. Hill, R. Lacroix, Y. Lee, T. G. van Leeuwen, N. Mackman, I. Mager, J. P. Nolan, E. van der Pol, D. M. Pegtel, S. Sahoo, P. R. M. Siljander, G. Sturk, O. de Wever y R. Nieuwland (2017). "Methodological guidelines to study extracellular vesicles." Circ Res **120**(10): 1632-1648.

Cuervo, A. M. y E. Wong (2014). "Chaperone-mediated autophagy: roles in disease and aging." Cell Res **24**(1): 92-104.

D'Acquisto, F., A. Merghani, E. Lecona, G. Rosignoli, K. Raza, C. D. Buckley, R. J. Flower y M. Perretti (2007). "Annexin-1 modulates T-cell activation and differentiation." Blood **109**(3): 1095-1102.

D'Souza-Schorey, C. y J. W. Clancy (2012). "Tumor-derived microvesicles: shedding light on novel microenvironment modulators and prospective cancer biomarkers." Genes Dev **26**(12): 1287-1299.

De Bari, C. y A. J. Roelofs (2018). "Stem cell-based therapeutic strategies for cartilage defects and osteoarthritis." Curr Opin Pharmacol **40**: 74-80.

Deregibus, M. C., V. Cantaluppi, R. Calogero, M. Lo Iacono, C. Tetta, L. Biancone, S. Bruno, B. Bussolati y G. Camussi (2007). "Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA." Blood **110**(7): 2440-2448.

Dokka, S., X. Shi, S. Leonard, L. Wang, V. Castranova y Y. Rojanasakul (2001). "Interleukin-10-mediated inhibition of free radical generation in macrophages." Am J Physiol Lung Cell Mol Physiol **280**(6): L1196-1202.

Domazetovic, V., G. Marcucci, T. Iantomasi, M. L. Brandi y M. T. Vincenzini (2017). "Oxidative stress in bone remodeling: role of antioxidants." Clin Cases Miner Bone Metab **14**(2): 209-216.

Dominici, M., K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop y E. Horwitz (2006). "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement." Cytotherapy **8**(4): 315-317.

Elmore, S. (2007). "Apoptosis: A review of programmed cell death." Toxicologic pathology **35**(4): 495-516.

Escrevente, C., S. Keller, P. Altevogt y J. Costa (2011). "Interaction and uptake of exosomes by ovarian cancer cells." BMC Cancer **11**: 108.

Ferlazzo, V., P. D'Agostino, S. Milano, R. Caruso, S. Feo, E. Cillari y L. Parente (2003). "Anti-inflammatory effects of annexin-1: stimulation of IL-10 release and inhibition of nitric oxide synthesis." Int Immunopharmacol **3**(10-11): 1363-1369.

Findlay, D. M. y G. J. Atkins (2014). "Osteoblast-chondrocyte interactions in osteoarthritis." Curr Osteoporos Rep **12**(1): 127-134.

Fisher, A. B. (2017). "Peroxiredoxin 6 in the repair of peroxidized cell membranes and cell signaling." Arch Biochem Biophys **617**: 68-83.

Fletcher, D. A. y R. D. Mullins (2010). "Cell mechanics and the cytoskeleton." Nature **463**(7280): 485-492.

Gebler, A., O. Zabel y B. Seliger (2012). "The immunomodulatory capacity of mesenchymal stem cells." Trends Mol Med **18**(2): 128-134.

Giai Via, A., A. Frizziero y F. Oliva (2012). "Biological properties of mesenchymal Stem Cells from different sources." Muscles, Ligaments and Tendons Journal **2**(3): 154-162.

Goekoop, R. J., M. Kloppenburg, H. M. Kroon, M. Frolich, T. W. Huizinga, R. G. Westendorp y J. Gussekloo (2010). "Low innate production of interleukin-1beta and interleukin-6 is associated with the absence of osteoarthritis in old age." Osteoarthritis Cartilage **18**(7): 942-947.

Goldring, M. B. y S. R. Goldring (2010). "Articular cartilage and subchondral bone in the pathogenesis of osteoarthritis." Ann N Y Acad Sci **1192**: 230-237.

Goldring, M. B. y K. B. Marcu (2009). "Cartilage homeostasis in health and rheumatic diseases." Arthritis Res Ther **11**(3): 224.

Goldring, M. B. y M. Otero (2011). "Inflammation in osteoarthritis." Curr Opin Rheumatol **23**(5): 471-478.

Goldring, M. B., M. Otero, D. A. Plumb, C. Dragomir, M. Favero, K. El Hachem, K. Hashimoto, H. I. Roach, E. Olivotto, R. M. Borzi y K. B. Marcu (2011). "Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis." Eur Cell Mater **21**: 202-220.

Gould, G. W. y J. Lippincott-Schwartz (2009). "New roles for endosomes: from vesicular carriers to multi-purpose platforms." Nat Rev Mol Cell Biol **10**(4): 287-292.

Gould, S. J. y G. Raposo (2013). "As we wait: coping with an imperfect nomenclature for extracellular vesicles." J Extracell Vesicles **2**. DOI: 10.3402/jev.v2i0.20389.

Greene, M. A. y R. F. Loeser (2015). "Aging-related inflammation in osteoarthritis." Osteoarthritis Cartilage **23**(11): 1966-1971.

Guescini, M., S. Genedani, V. Stocchi y L. F. Agnati (2010). "Astrocytes and glioblastoma cells release exosomes carrying mtDNA." J Neural Transm (Vienna) **117**(1): 1-4.

Hanson, P. I. y A. Cashikar (2012). "Multivesicular body morphogenesis." Annu Rev Cell Dev Biol **28**: 337-362.

Harding, C., J. Heuser y P. Stahl (1984). "Endocytosis and intracellular processing of transferrin and colloidal gold-transferrin in rat reticulocytes: demonstration of a pathway for receptor shedding." Eur J Cell Biol **35**(2): 256-263.

Haversath, M., I. Catelas, X. Li, T. Tassemeier y M. Jager (2012). "PGE(2) and BMP-2 in bone and cartilage metabolism: 2 intertwining pathways." Can J Physiol Pharmacol **90**(11): 1434-1445.

Headland, S. E. y L. V. Norling (2015). "The resolution of inflammation: Principles and challenges." Semin Immunol **27**(3): 149-160.

Henrotin, Y., B. Kurz y T. Aigner (2005). "Oxygen and reactive oxygen species in cartilage degradation: friends or foes?" Osteoarthritis Cartilage **13**(8): 643-654.

Itahana, K., J. Campisi y G. P. Dimri (2004). "Mechanisms of cellular senescence in human and mouse cells." Biogerontology **5**(1): 1-10.

Jaiswal, J. K., N. W. Andrews y S. M. Simon (2002). "Membrane proximal lysosomes are the major vesicles responsible for calcium-dependent exocytosis in nonsecretory cells." J Cell Biol **159**(4): 625-635.

Jevotovsky, D. S., A. R. Alfonso, T. A. Einhorn y E. S. Chiu (2018). "Osteoarthritis and stem cell therapy in humans: a systematic review." Osteoarthritis Cartilage.

Johnstone, R. M., M. Adam, J. R. Hammond, L. Orr y C. Turbide (1987). "Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes)." J Biol Chem **262**(19): 9412-9420.

Kapoor, M., J. Martel-Pelletier, D. Lajeunesse, J. P. Pelletier y H. Fahmi (2011). "Role of proinflammatory cytokines in the pathophysiology of osteoarthritis." Nat Rev Rheumatol **7**(1): 33-42.

Kassem, M. y P. J. Marie (2011). "Senescence-associated intrinsic mechanisms of osteoblast dysfunctions." Aging Cell **10**(2): 191-197.

Khosla, S., J. J. Westendorf y M. J. Oursler (2008). "Building bone to reverse osteoporosis and repair fractures." J Clin Invest **118**(2): 421-428.

Klumperman, J. y G. Raposo (2014). "The complex ultrastructure of the endolysosomal system." Cold Spring Harb Perspect Biol **6**(10): a016857.

Kobayashi, M., G. R. Squires, A. Mousa, M. Tanzer, D. J. Zukor, J. Antoniou, U. Feige y A. R. Poole (2005). "Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage." Arthritis Rheum **52**(1): 128-135.

Kolf, C. M., E. Cho y R. S. Tuan (2007). "Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation." Arthritis Research & Therapy **9**(1): 204-204.

Kowal, J., G. Arras, M. Colombo, M. Jouve, J. P. Morath, B. Primdal-Bengtson, F. Dingli, D. Loew, M. Tkach y C. Théry (2016). "Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes." Proc Natl Acad Sci U S A **113**(8): E968-977.

Lacroix, R., C. Judicone, M. Mooberry, M. Boucekine, N. S. Key y F. Dignat-George (2013). "Standardization of pre-analytical variables in plasma microparticle

determination: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop." J Thromb Haemost.

Lai, R. C., R. W. Yeo y S. K. Lim (2015). "Mesenchymal stem cell exosomes." Semin Cell Dev Biol **40**: 82-88.

Lane, R. E., D. Korbie, W. Anderson, R. Vaidyanathan y M. Trau (2015). "Analysis of exosome purification methods using a model liposome system and tunable-resistive pulse sensing." Sci Rep **5**: 7639.

Lasser, C., S. C. Jang y J. Lotvall (2018). "Subpopulations of extracellular vesicles and their therapeutic potential." Mol Aspects Med.

Le Blanc, K. y L. C. Davies (2018). "MSCs-cells with many sides." Cytotherapy **20**(3): 273-278.

Li, X., H. Afif, S. Cheng, J. Martel-Pelletier, J. P. Pelletier, P. Ranger y H. Fahmi (2005). "Expression and regulation of microsomal prostaglandin E synthase-1 in human osteoarthritic cartilage and chondrocytes." J Rheumatol **32**(5): 887-895.

Li, X., M. Ellman, P. Muddasani, J. H. Wang, G. Cs-Szabo, A. J. van Wijnen y H. J. Im (2009). "Prostaglandin E_2 and its cognate EP receptors control human adult articular cartilage homeostasis and are linked to the pathophysiology of osteoarthritis." Arthritis Rheum **60**(2): 513-523.

Liacini, A., J. Sylvester, W. Q. Li y M. Zafarullah (2002). "Inhibition of interleukin-1-stimulated MAP kinases, activating protein-1 (AP-1) and nuclear factor kappa B (NF-kappa B) transcription factors down-regulates matrix metalloproteinase gene expression in articular chondrocytes." Matrix Biol **21**(3): 251-262.

Liu, T., C. K. Daniels y S. Cao (2012). "Comprehensive review on the HSC70 functions, interactions with related molecules and involvement in clinical diseases and therapeutic potential." Pharmacol Ther **136**(3): 354-374.

Lobb, R. J., M. Becker, S. W. Wen, C. S. Wong, A. P. Wiegmans, A. Leimgruber y A. Moller (2015). "Optimized exosome isolation protocol for cell culture supernatant and human plasma." J Extracell Vesicles **4**: 27031.

Loeser, R. F. (2013). "Aging Processes and the Development of Osteoarthritis." Current opinion in rheumatology **25**(1): 108-113.

Loeser, R. F., S. R. Goldring, C. R. Scanzello y M. B. Goldring (2012). "Osteoarthritis: a disease of the joint as an organ." Arthritis Rheum **64**(6): 1697-1707.

Lotz, M. (1999). "The role of nitric oxide in articular cartilage damage." Rheum Dis Clin North Am **25**(2): 269-282.

Lv, F. J., R. S. Tuan, K. M. Cheung y V. Y. Leung (2014). "Concise review: the surface markers and identity of human mesenchymal stem cells." Stem Cells **32**(6): 1408-1419.

MacDonald, G. I., A. Augello y C. De Bari (2011). "Role of mesenchymal stem cells in reestablishing immunologic tolerance in autoimmune rheumatic diseases." Arthritis Rheum **63**(9): 2547-2557.

Manolagas, S. C. (2010). "From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis." Endocr Rev **31**(3): 266-300.

Martel-Pelletier, J., A. J. Barr, F. M. Cicuttini, P. G. Conaghan, C. Cooper, M. B. Goldring, S. R. Goldring, G. Jones, A. J. Teichtahl y J.-P. Pelletier (2016). "Osteoarthritis." Nature Reviews Disease Primers **2**: 16072.

Martel-Pelletier, J., L. M. Wildi y J. P. Pelletier (2012). "Future therapeutics for osteoarthritis." Bone **51**(2): 297-311.

Massicotte, F., D. Lajeunesse, M. Benderdour, J. P. Pelletier, G. Hilal, N. Duval y J. Martel-Pelletier (2002). "Can altered production of interleukin-1beta, interleukin-6, transforming growth factor-beta and prostaglandin E_2 by isolated human subchondral osteoblasts identify two subgroups of osteoarthritic patients." Osteoarthritis Cartilage **10**(6): 491-500.

Matthews, K. W., S. L. Mueller-Ortiz y R. A. Wetsel (2004). "Carboxypeptidase N: a pleiotropic regulator of inflammation." Mol Immunol **40**(11): 785-793.

Minghetti, L., A. Nicolini, E. Polazzi, A. Greco, M. Perretti, L. Parente y G. Levi (1999). "Down-regulation of microglial cyclo-oxygenase-2 and inducible nitric oxide synthase expression by lipocortin 1." Br J Pharmacol **126**(6): 1307-1314.

Minguzzi, M., S. Cetrullo, S. D'Adamo, Y. Silvestri, F. Flamigni y R. M. Borzi (2018). "Emerging players at the intersection of chondrocyte loss of maturational arrest, oxidative stress, senescence and low-grade inflammation in osteoarthritis." Oxid Med Cell Longev **2018**: 3075293.

Momen-Heravi, F., L. Balaj, S. Alian, P. Y. Mantel, A. E. Halleck, A. J. Trachtenberg, C. E. Soria, S. Oquin, C. M. Bonebreak, E. Saracoglu, J. Skog y W. P. Kuo (2013). "Current methods for the isolation of extracellular vesicles." Biol Chem **394**(10): 1253-1262.

Muralidharan-Chari, V., J. Clancy, C. Plou, M. Romao, P. Chavrier, G. Raposo y C. D'Souza-Schorey (2009). "ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles." Curr Biol **19**(22): 1875-1885.

Murphy, M. B., K. Moncivais y A. I. Caplan (2013). "Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine." Exp Mol Med **45**: e54.

Nabhan, J. F., R. Hu, R. S. Oh, S. N. Cohen y Q. Lu (2012). "Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein." Proc Natl Acad Sci U S A **109**(11): 4146-4151.

Nawaz, M., G. Camussi, H. Valadi, I. Nazarenko, K. Ekstrom, X. Wang, S. Principe, N. Shah, N. M. Ashraf, F. Fatima, L. Neder y T. Kislinger (2014). "The emerging role of extracellular vesicles as biomarkers for urogenital cancers." Nat Rev Urol **11**(12): 688-701.

Nery, A. A., I. C. Nascimento, T. Glaser, V. Bassaneze, J. E. Krieger y H. Ulrich (2013). "Human mesenchymal stem cells: from immunophenotyping by flow cytometry to clinical applications." Cytometry A **83**(1): 48-61.

Olivotto, E., M. Otero, K. B. Marcu y M. B. Goldring (2015). "Pathophysiology of osteoarthritis: canonical NF-κB/IKKbeta-dependent and kinase-independent effects of

IKKalpha in cartilage degradation and chondrocyte differentiation." RMD Open **1**(Suppl 1): e000061. DOI: 10.1136/rmdopen-2015-000061.

Osta, B., G. Benedetti y P. Miossec (2014). "Classical and Paradoxical Effects of TNF-α on Bone Homeostasis." Frontiers in Immunology **5**: 48.

Osteikoetxea, X., B. Sodar, A. Nemeth, K. Szabo-Taylor, K. Paloczi, K. V. Vukman, V. Tamasi, A. Balogh, A. Kittel, E. Pallinger y E. I. Buzas (2015). "Differential detergent sensitivity of extracellular vesicle subpopulations." Org Biomol Chem **13**(38): 9775-9782.

Ostergaard, O., C. T. Nielsen, L. V. Iversen, S. Jacobsen, J. T. Tanassi y N. H. Heegaard (2012). "Quantitative proteome profiling of normal human circulating microparticles." J Proteome Res **11**(4): 2154-2163.

Ostrowski, M., N. B. Carmo, S. Krumeich, I. Fanget, G. Raposo, A. Savina, C. F. Moita, K. Schauer, A. N. Hume, R. P. Freitas, B. Goud, P. Benaroch, N. Hacohen, M. Fukuda, C. Desnos, M. C. Seabra, F. Darchen, S. Amigorena, L. F. Moita y C. Thery (2010). "Rab27a and Rab27b control different steps of the exosome secretion pathway." Nat Cell Biol **12**(1): 19-30; sup pp 11-13.

Ouyang, N., C. Zhu, D. Zhou, T. Nie, M. F. Go, R. J. Richards y B. Rigas (2012). "MC-12, an annexin A1-based peptide, is effective in the treatment of experimental colitis." PLoS One **7**(7): e41585.

Page-McCaw, A., A. J. Ewald y Z. Werb (2007). "Matrix metalloproteinases and the regulation of tissue remodelling." Nat Rev Mol Cell Biol **8**(3): 221-233.

Paolini, L., A. Zendrini, G. Di Noto, S. Busatto, E. Lottini, A. Radeghieri, A. Dossi, A. Caneschi, D. Ricotta y P. Bergese (2016). "Residual matrix from different separation techniques impacts exosome biological activity." Sci Rep **6**: 23550.

Patel, T. R., D. J. Winzor y D. J. Scott (2016). "Analytical ultracentrifugation: A versatile tool for the characterisation of macromolecular complexes in solution." Methods **95**: 55-61.

Pedersen, K. W., B. Kierulf y A. Neurauter (2017). "Specific and generic isolation of extracellular vesicles with magnetic beads." Methods Mol Biol **1660**: 65-87.

Pérez, L. M., B. de Lucas, V. V. Lunyak y B. G. Gálvez (2017). "Adipose stem cells from obese patients show specific differences in the metabolic regulators vitamin D and Gas5." Molecular Genetics and Metabolism Reports **12**: 51-56.

Platas, J., M. I. Guillén, M. D. del Caz, F. Gomar, V. Mirabet y M. J. Alcaraz (2013). "Conditioned media from adipose-tissue-derived mesenchymal stem cells downregulate degradative mediators induced by interleukin-1beta in osteoarthritic chondrocytes." Mediators Inflamm **2013**: 357014.

Platas, J., M. I. Guillén, M. D. P. del Caz, F. Gomar, M. A. Castejón, V. Mirabet y M. J. Alcaraz (2016). "Paracrine effects of human adipose-derived mesenchymal stem cells in inflammatory stress-induced senescence features of osteoarthritic chondrocytes." Aging (Albany NY) **8**(8): 1703-1717.

Portal-Núñez, S., P. Esbrit, M. J. Alcaraz y R. Largo (2016). "Oxidative stress, autophagy, epigenetic changes and regulation by miRNAs as potential therapeutic targets in osteoarthritis." Biochem Pharmacol **108**: 1-10.

Quezada, C., A. Torres, I. Niechi, D. Uribe, S. Contreras-Duarte, F. Toledo, R. San Martin, J. Gutierrez y L. Sobrevia (2017). "Role of extracellular vesicles in glioma progression." Mol Aspects Med.

Rai, V., M. F. Dilisio, N. E. Dietz y D. K. Agrawal (2017). "Recent strategies in cartilage repair: A systemic review of the scaffold development and tissue engineering." J Biomed Mater Res A **105**(8): 2343-2354.

Raik, S., A. Kumar y S. Bhattacharyya (2018). "Insights into cell-free therapeutic approach: Role of stem cell "soup-ernatant"." Biotechnol Appl Biochem **65**(2): 104-118.

Ramírez, M. I., M. G. Amorim, C. Gadelha, I. Milic, J. A. Welsh, V. M. Freitas, M. Nawaz, N. Akbar, Y. Couch, L. Makin, F. Cooke, A. L. Vettore, P. X. Batista, R. Freezor, J. A. Pezuk, L. Rosa-Fernandes, A. C. O. Carreira, A. Devitt, L. Jacobs, I. T. Silva, G. Coakley, D. N. Nunes, D. Carter, G. Palmisano y E. Dias-Neto (2018). "Technical challenges of working with extracellular vesicles." Nanoscale **10**(3): 881-906.

Raposo, G., H. W. Nijman, W. Stoorvogel, R. Liejendekker, C. V. Harding, C. J. Melief y H. J. Geuze (1996). "B lymphocytes secrete antigen-presenting vesicles." J Exp Med **183**(3): 1161-1172.

Raposo, G. y W. Stoorvogel (2013). "Extracellular vesicles: Exosomes, microvesicles, and friends." J Cell Biol **200**(4): 373-383.

Ratajczak, J., K. Miekus, M. Kucia, J. Zhang, R. Reca, P. Dvorak y M. Z. Ratajczak (2006). "Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery." Leukemia **20**(5): 847-856.

Ratajczak, M. Z., T. Jadczyk, D. Pedziwiatr y W. Wojakowski (2014). "New advances in stem cell research: practical implications for regenerative medicine." Pol Arch Med Wewn **124**(7-8): 417-426.

Reinisch, A., N. Etchart, D. Thomas, N. A. Hofmann, M. Fruehwirth, S. Sinha, C. K. Chan, K. Senarath-Yapa, E. Y. Seo, T. Wearda, U. F. Hartwig, C. Beham-Schmid, S. Trajanoski, Q. Lin, W. Wagner, C. Dullin, F. Alves, M. Andreeff, I. L. Weissman, M. T. Longaker, K. Schallmoser, R. Majeti y D. Strunk (2015). "Epigenetic and in vivo comparison of diverse MSC sources reveals an endochondral signature for human hematopoietic niche formation." Blood **125**(2): 249-260.

Ren, G., X. Zhao, L. Zhang, J. Zhang, A. L'Huillier, W. Ling, A. I. Roberts, A. D. Le, S. Shi, C. Shao y Y. Shi (2010). "Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression." J Immunol **184**(5): 2321-2328.

Roberts, C. T., Jr. y P. Kurre (2013). "Vesicle trafficking and RNA transfer add complexity and connectivity to cell-cell communication." Cancer Res **73**(11): 3200-3205.

Roberts, G. S., D. Kozak, W. Anderson, M. F. Broom, R. Vogel y M. Trau (2010). "Tunable nano/micropores for particle detection and discrimination: scanning ion occlusion spectroscopy." Small **6**(23): 2653-2658.

Rosa-Fernandes, L., V. B. Rocha, V. C. Carregari, A. Urbani y G. Palmisano (2017). "A perspective on extracellular vesicles proteomics." Front Chem **5**: 102.

Sheikh, M. H. y E. Solito (2018). "Annexin A1: Uncovering the many talents of an old protein." Int J Mol Sci **19**(4).

Shelke, G. V., C. Lässer, Y. S. Gho y J. Lötvall (2014). "Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum." Journal of Extracellular Vesicles **3**. DOI: 10.3402/jev.v3403.24783.

Six, D. A. y E. A. Dennis (2000). "The expanding superfamily of phospholipase A(2) enzymes: classification and characterization." Biochim Biophys Acta **1488**(1-2): 1-19.

Sodar, B. W., A. Kittel, K. Paloczi, K. V. Vukman, X. Osteikoetxea, K. Szabo-Taylor, A. Nemeth, B. Sperlagh, T. Baranyai, Z. Giricz, Z. Wiener, L. Turiak, L. Drahos, E. Pallinger, K. Vekey, P. Ferdinandy, A. Falus y E. I. Buzas (2016). "Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection." Sci Rep **6**: 24316.

Sowerby, S. J., M. F. Broom y G. B. Petersen (2007). "Dynamically resizable nanometre-scale apertures for molecular sensing." Sensors and Actuators B: Chemical **123**(1): 325-330.

Stranska, R., L. Gysbrechts, J. Wouters, P. Vermeersch, K. Bloch, D. Dierickx, G. Andrei y R. Snoeck (2018). "Comparison of membrane affinity-based method with size-exclusion chromatography for isolation of exosome-like vesicles from human plasma." Journal of Translational Medicine **16**(1): 1.

Stuffers, S., C. Sem Wegner, H. Stenmark y A. Brech (2009). "Multivesicular endosome biogenesis in the absence of ESCRTs." Traffic **10**(7): 925-937.

Sundararajan, V., F. H. Sarkar y T. S. Ramasamy (2018). "The versatile role of exosomes in cancer progression: diagnostic and therapeutic implications." Cell Oncol (Dordr). **41**(3): 223-252.

Szatanek, R., M. Baj-Krzyworzeka, J. Zimoch, M. Lekka, M. Siedlar y J. Baran (2017). "The methods of choice for extracellular vesicles (EVs) characterization." Int J Mol Sci **18**(6).

Tai, H., C. Miyaura, C. C. Pilbeam, T. Tamura, Y. Ohsugi, Y. Koishihara, N. Kubodera, H. Kawaguchi, L. G. Raisz y T. Suda (1997). "Transcriptional induction of cyclooxygenase-2 in osteoblasts is involved in interleukin-6-induced osteoclast formation." Endocrinology **138**(6): 2372-2379.

Taylor, D. D. y S. Shah (2015). "Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes." Methods **87**: 3-10.

Taylor, D. D., W. Zacharias y C. Gercel-Taylor (2011). "Exosome isolation for proteomic analyses and RNA profiling." Methods Mol Biol **728**: 235-246.

Tchkonia, T., Y. Zhu, J. van Deursen, J. Campisi y J. L. Kirkland (2013). "Cellular senescence and the senescent secretory phenotype: therapeutic opportunities." J Clin Invest **123**(3): 966-972.

Théry, C., S. Amigorena, G. Raposo y A. Clayton (2006). "Isolation and characterization of exosomes from cell culture supernatants and biological fluids." Curr Protoc Cell Biol **Chapter 3**: Unit 3.22.

Théry, C., M. Ostrowski y E. Segura (2009). "Membrane vesicles as conveyors of immune responses." Nat Rev Immunol **9**(8): 581-593.

Tian, J., F. J. Zhang y G. H. Lei (2015). "Role of integrins and their ligands in osteoarthritic cartilage." Rheumatol Int **35**(5): 787-798.

Tricarico, C., J. Clancy y C. D'Souza-Schorey (2017). "Biology and biogenesis of shed microvesicles." Small GTPases **8**(4): 220-232.

Valadi, H., K. Ekstrom, A. Bossios, M. Sjostrand, J. J. Lee y J. O. Lotvall (2007). "Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells." Nat Cell Biol **9**(6): 654-659.

van Buul, G. M., E. Villafuertes, P. K. Bos, J. H. Waarsing, N. Kops, R. Narcisi, H. Weinans, J. A. Verhaar, M. R. Bernsen y G. J. van Osch (2012). "Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explant culture." Osteoarthritis Cartilage **20**(10): 1186-1196.

van der Vorst, E. P. C., R. J. de Jong y M. Donners (2018). "Message in a microbottle: modulation of vascular inflammation and atherosclerosis by extracellular vesicles." Front Cardiovasc Med **5**: 2.

van Eijndhoven, M. A., J. M. Zijlstra, N. J. Groenewegen, E. E. Drees, S. van Niele, S. R. Baglio, D. Koppers-Lalic, H. van der Voorn, S. F. Libregts, M. H. Wauben, R. X. de Menezes, J. R. van Weering, R. Nieuwland, L. Visser, A. van den Berg, D. de Jong y D. M. Pegtel (2016). "Plasma vesicle miRNAs for therapy response monitoring in Hodgkin lymphoma patients." JCI Insight **1**(19): e89631.

van Niel, G., G. D'Angelo y G. Raposo (2018). "Shedding light on the cell biology of extracellular vesicles." Nat Rev Mol Cell Biol **19**(4): 213-228.

Vidal, M. J. y P. D. Stahl (1993). "The small GTP-binding proteins Rab4 and ARF are associated with released exosomes during reticulocyte maturation." Eur J Cell Biol **60**(2): 261-267.

Vogel, R., F. A. Coumans, R. G. Maltesen, A. N. Boing, K. E. Bonnington, M. L. Broekman, M. F. Broom, E. I. Buzas, G. Christiansen, N. Hajji, S. R. Kristensen, M. J. Kuehn, S. M. Lund, S. L. Maas, R. Nieuwland, X. Osteikoetxea, R. Schnoor, B. J. Scicluna, M. Shambrook, J. de Vrij, S. I. Mann, A. F. Hill y S. Pedersen (2016). "A standardized method to determine the concentration of extracellular vesicles using tunable resistive pulse sensing." J Extracell Vesicles **5**: 31242.

Whitehead, B., L. Wu, M. L. Hvam, H. Aslan, M. Dong, L. Dyrskjøt, M. S. Ostenfeld, S. M. Moghimi y K. A. Howard (2015). "Tumour exosomes display differential mechanical and complement activation properties dependent on malignant state: implications in endothelial leakiness." Journal of Extracellular Vesicles **4**: 29685. DOI: 10.3402/jev.v3404.29685.

Whiteside, T. L. (2017). "Extracellular vesicles isolation and their biomarker potential: are we ready for testing?" Annals of Translational Medicine **5**(3): 54.

Witwer, K. W., E. I. Buzás, L. T. Bemis, A. Bora, C. Lasser, J. Lotvall, E. N. Nolte-'t Hoen, M. G. Piper, S. Sivaraman, J. Skog, C. Théry, M. H. Wauben y F. Hochberg (2013). "Standardization of sample collection, isolation and analysis methods in extracellular vesicle research." J Extracell Vesicles **2**. DOI: 10.3402/jev.v2i0.20360.

Wolf, P. (1967). "The nature and significance of platelet products in human plasma." Br J Haematol **13**(3): 269-288.

Wyatt, A. R., J. H. Cater y M. Ranson (2016). "PZP and PAI-2: Structurally-diverse, functionally similar pregnancy proteins?" Int J Biochem Cell Biol **79**: 113-117.

Xu, J. F., G. H. Yang, X. H. Pan, S. J. Zhang, C. Zhao, B. S. Qiu, H. F. Gu, J. F. Hong, L. Cao, Y. Chen, B. Xia, Q. Bi y Y. P. Wang (2014). "Altered microRNA expression profile in exosomes during osteogenic differentiation of human bone marrow-derived mesenchymal stem cells." PLoS One **9**(12): e114627.

Yáñez-Mo, M., P. R. Siljander, Z. Andreu, A. B. Zavec, F. E. Borrás, E. I. Buzás, K. Buzás, E. Casal, F. Cappello, J. Carvalho, E. Colas, A. Cordeiro-da Silva, S. Fais, J. M. Falcón-Pérez, I. M. Ghobrial, B. Giebel, M. Gimona, M. Graner, I. Gursel, M. Gursel, N. H. Heegaard, A. Hendrix, P. Kierulf, K. Kokubun, M. Kosanovic, V. Kralj-Iglic, E. M. Kramer-Albers, S. Laitinen, C. Lasser, T. Lener, E. Ligeti, A. Line, G. Lipps, A. Llorente, J. Lotvall, M. Mancek-Keber, A. Marcilla, M. Mittelbrunn, I. Nazarenko, E. N. Nolte-'t Hoen, T. A. Nyman, L. O'Driscoll, M. Olivan, C. Oliveira, E. Pallinger, H. A. Del Portillo, J. Reventos, M. Rigau, E. Rohde, M. Sammar, F. Sánchez-Madrid, N. Santarem, K. Schallmoser, M. S. Ostenfeld, W. Stoorvogel, R. Stukelj, S. G. Van der Grein, M. H. Vasconcelos, M. H. Wauben y O. De Wever (2015). "Biological properties of extracellular vesicles and their physiological functions." J Extracell Vesicles **4**: 27066. DOI: 10.3402/jev.v4.27066.

Zuk, P. A., M. Zhu, H. Mizuno, J. Huang, J. W. Futrell, A. J. Katz, P. Benhaim, H. P. Lorenz y M. H. Hedrick (2001). "Multilineage cells from human adipose tissue: implications for cell-based therapies." Tissue Eng **7**(2): 211-228.

ANEXO I

Comparative optimization of extracellular vesicle isolation procedures in the development of new therapeutic strategies for osteoarthritis

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Draft

Introduction

The use of extracellular vesicles (EVs) for therapeutic purposes has risen growing interest among the scientific community in disparate areas of the biomedical sciences, from rheumatology to oncology. In particular, the use of EVs from the conditioned medium (CM) of mesenchymal stem cells as a cell-free therapy may present advantages in treating inflammatory conditions, due to its increased simplicity and lower risk of negative secondary effects such as carcinogenesis (Bacakova, Zarubova et al. 2018). However, this potential is crucially dependent on the development of standardized isolation methods. An ideal isolation method should deplete most protein and lipid contaminants, such as plasma proteins and lipoproteins, while yielding a sufficient quantity of EVs for their downstream molecular and functional analysis, as well as being reproducible, effective and simple to perform, especially if it is to be scaled up to clinical practice. Currently, there is not such a method available (Coumans, Brisson et al. 2017).

The isolation of both small- (exosomes or EX) and medium-sized (microvesicles or MV) EVs from CM presents several problems related to the medium high viscosity and presence of high amounts of serum proteins and lipids. In addition, it usually requires at least one step of pre-concentration (Coumans, Brisson et al. 2017). If the use of serum-free media is not possible, the depletion of serum EVs and abundant proteins is mandatory to avoid bias in downstream analysis (Lacroix, Judicone et al. 2013, Shelke, Lässer et al. 2014). Once isolated, sample handling is crucial in order to avoid EV alteration or degradation. In particular, freezing is recommended at -80ºC or liquid nitrogen, and successive freeze-thaw cycles may severely compromise EV integrity (Coumans, Brisson et al. 2017).

The most popular method for the isolation of EVs is differential centrifugation, which involves centrifugation at increasing speeds to separate EVs according to their size-dependent weight. Each centrifugation is usually preceded by size-filtering to eliminate contaminants (Carpintero-Fernández, Fafián-Labora et al. 2017). However, this method has several limitations, such as EV aggregation, compromised EV integrity and the presence of protein impurities; as well as various impracticabilities, such as long working times or the need of specialized equipment (Lobb, Becker et al. 2015, Stranska, Gysbrechts et al. 2018). To increase sample purity, additional washing steps may be coupled, but successive centrifugations affect the EV integrity and yield.

Size exclusion chromatography (SEC), on the other hand, has been shown to work well in separating EVs from high-density lipoproteins and proteins (Lobb, Becker et al. 2015) and has been used successfully for small scale analysis as well as near-to clinical practice (Boing, van der Pol et al. 2014, van Eijndhoven, Zijlstra et al. 2016). However, despite its great capacity to isolate relatively uncontaminated EVs, SEC has several technical and practical limitations. First, it only allows the efficient isolation of EVs with a size larger than the pore and the matrix size used (70 nm for CL-2B Sepharose), and does not allow EV subfractioning. Also, some contaminants may remain, such as low density lipoproteins and chylomicrons (Sodar, Kittel et al. 2016). On the other hand, sample and equipment handling may introduce variability (Boing, van der Pol et al. 2014).

Several polymers, polyethylene glycol (PEG) in particular, have been used to isolate viruses and other macromolecules for more than 50 years. Throughout the last decade, several commercial polymers have been developed, most of which are derived from PEG solutions, to isolate EVs (Momen-Heravi, Balaj et al. 2013). The best known and most used is ExoQuick™ from System Biosciences. The use of these polymers is technically easy, does not require specialized equipment and involves very short protocol times. However, the method co-precipitates protein complexes and circulating RNA in high proportions, which make downstream analysis difficult (Taylor, Zacharias et al. 2011).

In this work, we have compared the performance of differential centrifugation, using the SW28 ultracentrifugation rotor (Beckman Coulter), SEC with 10 mL qEV columns (Izon Science) and ExoQuick™ for the isolation of EVs from 30 mL of CM obtained from adipose tissue-derived mesenchymal stem cells (ASCs), in terms of medium concentration and size distribution by tunable resistive pulse sensing (TRPS) with the qNano equipment of Izon Science. Finally, we have assessed the pharmacological potential of the EVs obtained to treat primary cultures of interleukin (IL)-1β stimulated human osteoarthritic chondrocytes.

Material and methods

Tissues, Cells and Culture Media

ASCs were obtained from the adipose tissue of 10 healthy donors (3 men and 7 women, aged 51.3 ± 6.8 years, mean ± SEM) who had undergone abdominoplasty. Samples were washed with phosphate-buffered saline (PBS), minced and digested at 37ºC for 1 h with 10% of type I collagenase (Gibco, Life Technologies, Madrid, Spain). Tissue remains were filtered through a 100 µm cell strainer (BD Biosciences, Durham, NC, USA). Then, cells were washed with DMEM/HAM F12 (Sigma-Aldrich, St. Luis, MO, USA) containing penicillin and streptomycin (1%), seeded onto tissue culture flasks ($1–2\times10^6$ cells/mL, 30 ml culture) in the same media supplemented with 15% EV-free human serum, and incubated at 37ºC in a 5% CO_2 atmosphere. Human serum was obtained from whole-blood donations of AB-blood-group-typed donors according to the criteria of Valencia Transfusion Centre, Spain. To eliminate EV fraction, serum was centrifuged during 18 h at 120,000g and 4ºC using a SW-28 swinging-bucket rotor (Beckman Coulter, Brea, CA, USA). At 24 h, when cells reached semiconfluence, culture plates were washed and the ASC phenotype confirmed by flow cytometry (BD FACSVerse™ flow cytometer o BD LSRFortessa™ cell analyzer, BD Biosciences) using specific antibodies: Phycoerythrin (PE)-conjugated mouse monoclonal antibody (mAb) against human CD105 (Endoglin, clone SN6, IgG1), PerCP-eFluor™ 710-conjugated mouse mAb against human CD90 (Thy-1, clone eBio5E10 (5E10), IgG1), Allophycocyanin (APC)-conjugated mouse mAb against human CD34 (clone 4H11, IgG1) (eBioscience™, Inc., San Diego, CA, USA), and PE-conjugated mouse mAb against human CD45 (clone HI30, IgG1) (BD Biosciences) and measuring cellular viability with propidium iodide.

CM was collected at passage 0 after 48 h of culture. It was pooled, centrifuged, and stored in sterile conditions at −80º C prior to further use.

Knee specimens were obtained from patients diagnosed with advanced osteoarthritis (12 women and 6 men, aged 64.3 ± 9.7 years, mean ± SEM) who had undergone a total joint replacement. Diagnosis was based on clinical, laboratory, and radiological evaluation. For primary cell isolation, cartilage was dissected from the femoral condyles and tibial plateau of the knee joint and diced into small pieces. Human articular chondrocytes were isolated by sequential enzymatic digestion: 1 h with 0.1 mg/mL hyaluronidase (Sigma-Aldrich) followed by 12–15 h with 2 mg/mL type IA collagenase (Sigma-Aldrich) in

DMEM/HAM F12 (Sigma-Aldrich) containing penicillin and streptomycin (1%) at 37ºC in 5% CO_2 atmosphere. The digested tissue was filtered through a 70 µm nylon mesh (BD Biosciences), washed, and centrifuged. Cell viability was measured and attested as greater than 95% according to the Trypan Blue (Sigma-Aldrich) exclusion test. All experiments were performed with chondrocyte primary cultures at semiconfluence (270×10^3 cells/well in 6-well plates or 1.5×10^6 cells in 3.5 cm plates). Chondrocytes were maintained with 5% CO_2 at 37ºC in DMEM/HAM F12 (Sigma-Aldrich) containing penicillin and streptomycin (1%), supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich).

For stimulation, chondrocytes were incubated for 24h with 10 mg/mL IL-1β in DMEM/HAM F12 (Sigma-Aldrich) containing penicillin and streptomycin (1%) supplemented with 15% EV-free human serum, and treated with different concentrations of MV or EX for 24 h.

The experimental design was approved by the Institutional Ethical Committees (Universitat de València and Hospital Universitari i Politècnic La Fe, Valencia, Spain). Samples were obtained from donors after they provided informed consent according to the Helsinki Declaration of 1975, as revised in 2013.

Vesicle isolation by one-step precipitation

Single-step precipitation of EV samples was performed with the ExoQuick™-TC reagent (System Biosciences LLC, Palo Alto, CA, USA) as specified by the manufacturer. Briefly, 1 mL of CM was centrifuged at 3,000g for 15 min to remove cells and cell debris. Next, supernatants were transferred to a sterile microtube and mixed with 252 µL of the ExoQuick reagent by flicking the tubes. Samples were incubated at 4ºC for 30 min with the tubes upright and without rotation. Next, mixtures were centrifuged at 1,500g for 30 min at 4ºC. Supernatant was collected and residual ExoQuick solution spinned down by centrifugation at 1,500g for 5 min. All fluid traces were removed and the pellet was resuspended in 100 µL of sterile PBS. Samples were then analysed fresh or stored at -80ºC until further use.

Vesicle isolation by size exclusion chromatography

Separation of EV fractions by SEC was done by means of the qEV-original columns (IZON Sciences Ltd, Oxford, UK) as specified by the manufacturer. These columns contain a resin with a pore size of around 75 nm, a bed volume of 10 mL and height of

51 mm, an inner tube diameter of 15.6 mm, a void volume of 3.0 ± 0.25 mL, a sample volume of ideally 500 µL with flow rates of typically 0.8–1.2 mL/min at room temperature. Columns are pre-filled with PBS, containing 0.05% sodium azide (Vogel, Coumans et al. 2016).

First, columns were vertically immobilized and rinsed with sterile PBS. 500 µL of CM were pipetted onto the column. Sterile PBS was sequentially added to avoid column drying. After dead volume elution, 500 µL fractions 6 up to 11 were collected in sterile microtubes, as fractions 7-9 usually contain most of EV (Boing, van der Pol et al. 2014). Fractions were then analysed fresh or stored at -80ºC until further use.

Vesicle isolation by differential centrifugation

Cells and cellular debris were eliminated by pelleting with centrifugation at 300g for 10 min. Supernatants were pre-filtered with a 40 µm filter (Merck, Darmstadt, Germany) to remove remnants of large structures. Vesicles were then collected from the supernatant through differential centrifugation steps. All filtration steps were accomplished solely by gravity, letting the fluid go down drop by drop. CM was filtered first through 5 µm filter (Merck, New Jersey, USA) and centrifuged at 3,000g for 10 min to pellet apoptotic bodies. The supernatants were then filtered through 800 nm filter (Merck) at 4ºC or room temperature and centrifuged at 12,200g for 20 min at 4°C or room temperature to pellet MV. Finally, supernatants were filtered through 200 nm filter (Merck) at 4ºC or room temperature and centrifuged at 100,000g for 90 min with a SW28 Ti Rotor (Beckman Coulter) at 4ºC or room temperature. EV pellets were washed once with sterile PBS, resuspended in 15 µL of PBS and analysed fresh or stored at -80ºC until further use.

Tunable Resistive Pulse Sensing (TRPS)

EV preparations were analysed by TRPS using a qNano instrument (IZON Sciences Ltd.) as previously described (Vogel, Coumans et al. 2016). Except from apoptotic bodies, EVs were studied fresh and after 1, 2 and 3 successive freeze-thaw cycles at -80ºC. PBS-containing surfactant (0.03% Tween 20) was used to prevent spontaneous EV aggregation in solution. TRPS measurements are more stable and accurate when surfactant is present in the buffer (Anderson, Lane et al. 2015). As higher than 0.05% Tween 20 may disrupt the EV membrane and the total EV concentration is unaffected up to concentrations of 5% Tween 20 (Osteikoetxea, Sodar et al. 2015), we used a lower 0.03% Tween 20 to

sufficiently prevent EV aggregation but not disrupt EV structure or morphology (Vogel, Coumans et al. 2016).

Surfactant-containing PBS was placed in both fluid cells with an electrode each (below and above the nanopore). Calibration was performed using calibration beads CPC100, SKP400 and CPC2000, provided by the manufacturer (IZON Sciences Ltd.). Nanopore stretch, voltage and applied pressure were adjusted on both calibration samples, so that the relative blockade magnitude was within 0.0025–0.005 and the inverse full-width half maximum duration, which reflects particle speed, was within 10–15/ms. By correctly stretching the nanopore and fine-tuning the voltage, particles were measured in the detection range. Then, NP100, NP300 and NP2000 nanopore membranes were used to measure the samples of EXs, MVs and apoptotic bodies, respectively. At least 500 events/sample were counted. Data collection and analysis was performed with the Izon Control Suite (v3.1, IZON Sciences Ltd.) software.

Protein quantification

Protein content was quantified with the DC™ Protein Assay (Bio-Rad, Hercules, CA, USA) as specified by the manufacturer. Working reagent was prepared by adding 20 µL of reagent S to 1 mL of reagent A. Then, a serial dilution of bovine serum albumin (BSA), as protein standard, was prepared with concentrations from 0.2 mg/mL to 1.5 mg/mL. 5 µL of standards and samples were pipetted into a clean, dry microwell plate. 25 µL of working reagent and then 200 µL reagent B were added into each well. The plate was gently agitated to mix reagents and samples. After 15 minutes, absorbance was measured at 750 nm with a Victor3 microplate reader (PerkinElmer, Waltham, MA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Chondrocytes were stimulated with IL-1β (10 ng/mL) in presence or absence of different concentrations of MVs ($6x10^6$, $1.2x10^7$, $2.4x10^7$, $3.6x10^7$ and $6x10^7$ particles/mL) or EXs ($5x10^6$, 10^7, $1.5x10^7$, $7.2x10^7$ and 10^8 particles/mL) for 24h. Supernatants were harvested, centrifuged, and frozen at -80ºC until analysis. TNFα and IL-6 were measured from supernatans with ELISA kits from eBioscience with a sensitivity of 4.0 pg/mL for both proteins.

Statistical Analysis

The data were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-test using the GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Values were expressed as individual data points, percentages or mean ± standard error of the mean (SEM) when appropriate. A *P* value of less than 0.05 was considered to be significant.

Results and discussion

1. Single-step precipitation performed poorly and did not allow TRPS analysis

Precipitation of biological structures by polymer conjugation has been widespread used in different experimental contexts. It has several advantages such as its quickness and simplicity. However, there are increasing concerns about its usefulness for isolating EVs from highly complex fluids such as plasma or culture media. First, EVs and specially EXs tend to aggregate, which makes downstream analysis difficult. Second, many proteins circulate loosely associated with EV membranes or forming affinity complexes around them. Single-step precipitation co-precipitates many of these proteins with them, resulting in impure isolates which are difficult to analyse, quantify or sub-fractionate (Coumans, Brisson et al. 2017).

After single-step precipitation with ExoQuick™, our EV preparations blocked the NP300 nanopore used to analyse them, completely preventing further studies. Serial dilutions were done and the efflux through the pore was possible to rescue, but diluted samples were unable to provide consistent measures (not shown). Hence, single-step precipitation was discarded for further analysis.

2. Size exclusion chromatography eliminated most protein contaminants but yielded low EV concentrations

SEC separated particles in different PBS-collected fractions. The biggest concentration of small sized EVs was found in fraction 10, whereas medium sized EVs eluted around fraction 9 (Fig. 1). However, fractionation did not separate EV subpopulations successfully. Also, EV concentration was low in every fraction, and a sufficient yield may require a pre-concentration by centrifugation or ultrafiltration (Coumans, Brisson et al. 2017).

On the other hand, the protein amount detected in the fraction supernatants was the lowest of all isolation procedures, as shown in figure 1. Most proteins eluted from fraction 10 onwards. EV relevant fractions yielded a low amount of total proteins.

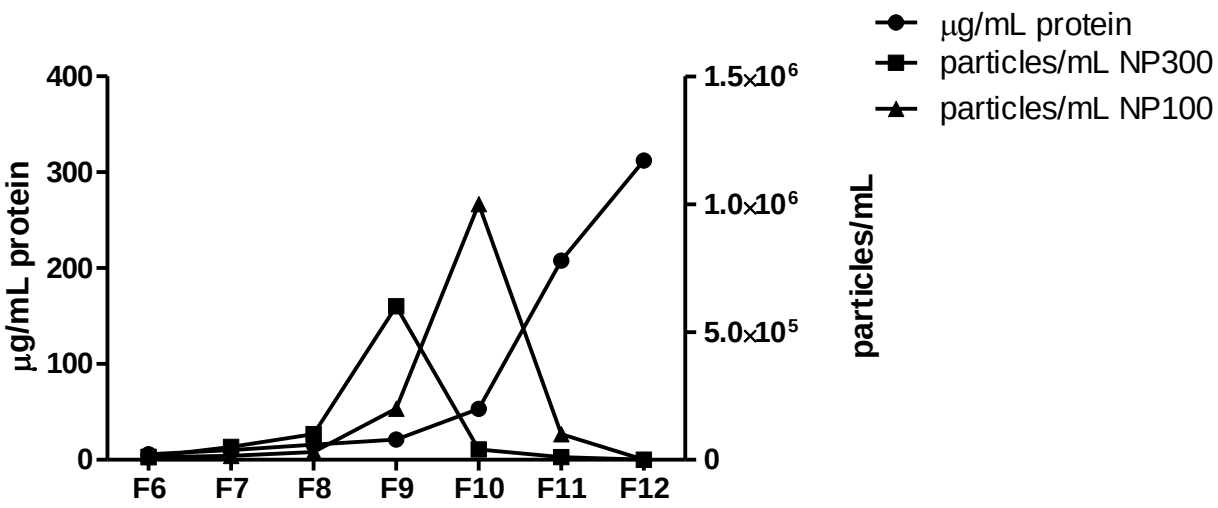

Figure 1. Supernatant protein concentration and EV concentration measured by DCTM Protein Assay (Bio-Rad) and TRPS (qNano, Izon Science) respectively. Medium-sized EVs/MVs were measured by means of the NP300 nanopore, whereas small-sized EVs/EXs were measured with the NP100 nanopore, using SKP400 and CPC100 calibration particles, respectively.

3. Differential centrifugation yielded the highest EV concentration and allowed subpopulation enrichment

The combination of filtration with differential centrifugation allowed the pelleting of MV and EX subpopulation separately, as well as apoptotic bodies. TRPS analysis revealed a raw concentration of $7.2*10^5$ particles/mL for apoptotic bodies, $8*10^9$ particles/mL for MVs, $3.8*10^{10}$ particles/mL for EXs and; and an average diameter of 1947, 316 and 115 nm, respectively (Fig. 2a). Due to their low concentration, apoptotic bodies were discarded for further analysis.

Presence of protein contaminants in MV- and EX-enriched isolates was much higher than in SEC. This concentration was reduced when MVs and EXs were washed once with PBS (Fig. 2b). This indicates that differential centrifugation may be the best suited method of those studied here to isolate EVs from CM in our experimental conditions.

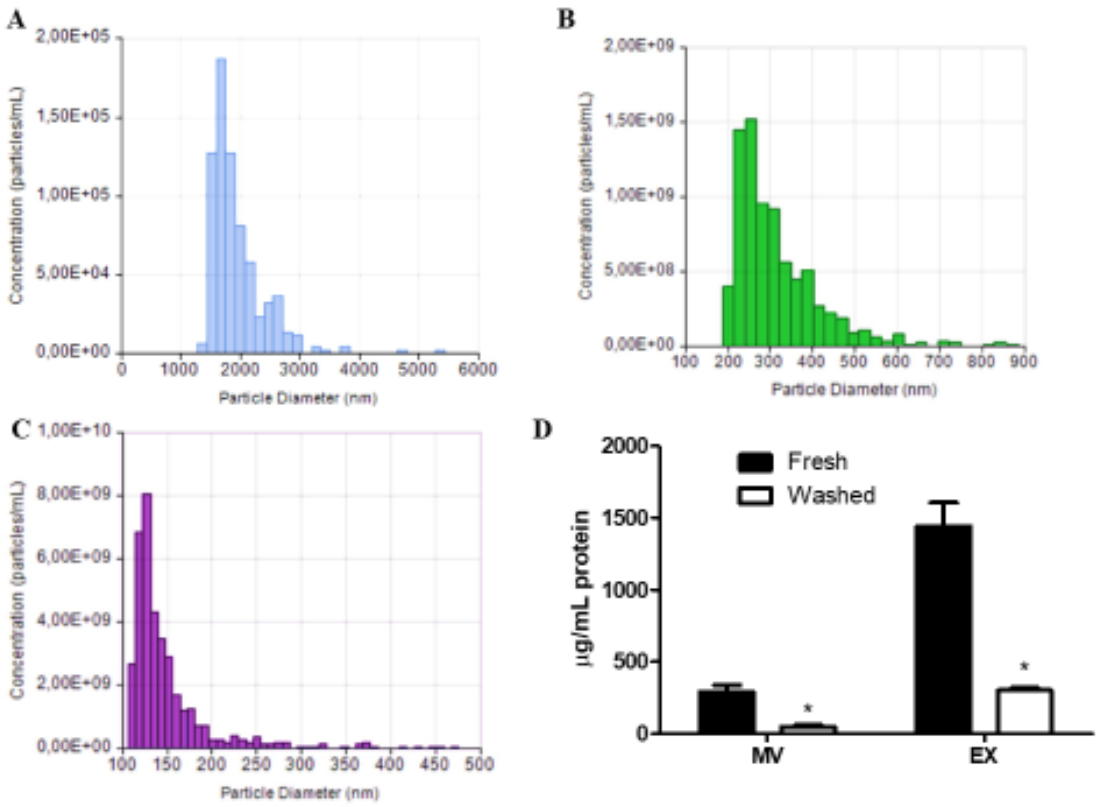

Figure 2. Representative TRPS analysis of **(A)** apoptotic bodies, **(B)** MVs and **(C)** EXs. **(D)** Protein concentration in MV and EX supernatant both fresh and after being washed with PBS once. *p<0.05 respect to fresh.

4. Centrifugation temperature, but not filtration, had a negative effect on the EV concentration

As differential centrifugation yielded the highest EV concentration and allowed subpopulation separation, we focused on this method and assessed several relevant experimental conditions in it, such as assay temperature, freezing influence and biological activity.

As seen in figure 3a, a low centrifugation temperature for pelleting both MVs and EXs resulted in an increased yield in terms of particles/mL. A decrease in the EV concentration at higher centrifugation times may be due to shearing forces which could disrupt EV integrity (Coumans, Brisson et al. 2017).

Interestingly, temperature during filtration did not affect EV concentration neither in MVs nor EXs (Fig. 3b). As viscosity is inversely correlated with temperature, several authors have suggested that decreased temperature during filtration steps may hinder the gravity-induced flow of vesicles and other particles downwards (Patel, Winzor et al. 2016). Of note, filtration time was much higher when working at low temperatures.

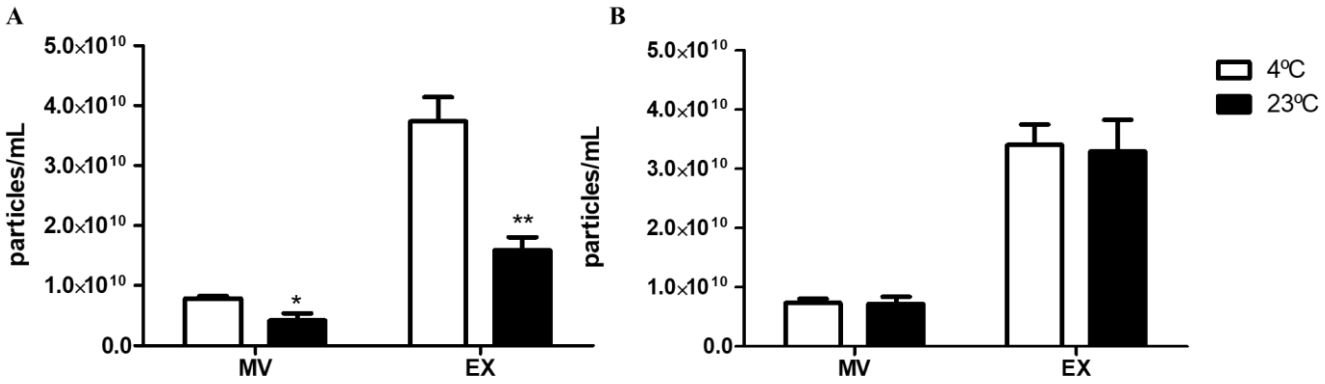

Figure 3. Effect of temperature on particle concentration during **(A)** centrifugation and **(B)** filtration in the course of the filtration-ultracentrifugation process. *p<0.05; **p<0.01 respect to 4ºC.

5. Successive freeze-thaw cycles significantly compromised EV integrity

As expected (Coumans, Brisson et al. 2017), an increase in the number of freeze-thaw cycles critically reduced the concentration of both types of vesicles, expressed as particles/mL (Fig. 4). The formation of membrane-disrupting ice crystals has been proposed as the main contributor to this effect. Interestingly, however, the first freeze-thaw cycle did not significantly affect EV integrity in either subpopulation. This may be of clinical relevance, as sample handling and storage is critical in a therapeutic context, and avoiding the need of fresh samples for therapeutic purposes could make EV-based therapies more feasible in a medium-term context.

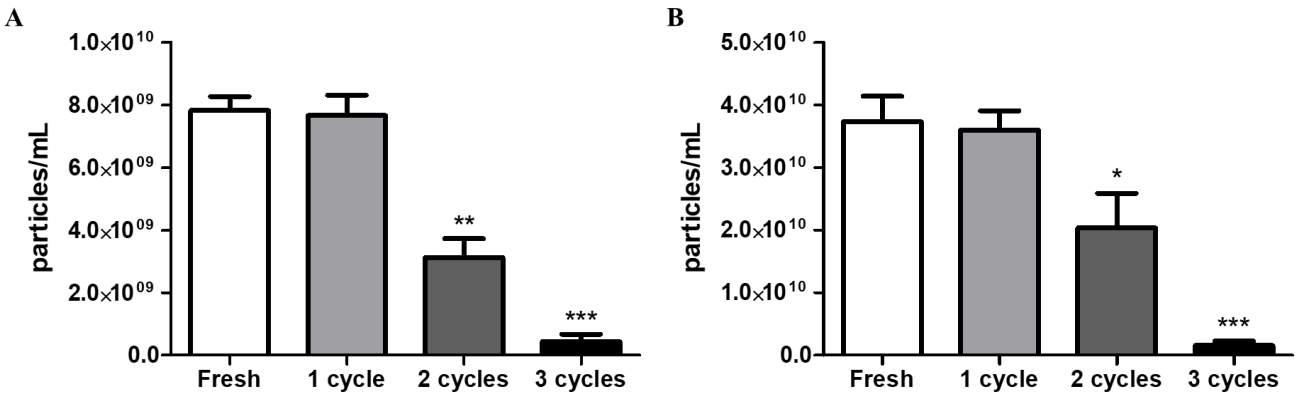

Figure 4. Effect of freeze-thaw cycles on particle concentration after an isolation by filtration-centrifugation. *p<0.05; **p<0.01; ***p<0.001 respect to fresh.

6. Dose-response curves were successfully established for both MVs and EXs

OA primary chondrocytes were stimulated by incubation with IL-1β in presence or absence of MVs and EXs. Stimulation of OA chondrocytes with IL-1β for 24 h increased the release of the pro-inflammatory cytokines IL-6 and TNFα into the culture medium compared with control nonstimulated cells (Figs. 5 and 6). Chondrocytes were treated with MVs or EXs at increasing concentrations, and significantly reduced the liberation of both proinflammatory cytokines to the supernatant. Optimal pharmacological concentrations were established for MV-enriched ($3.6*10^7$ particles/mL) and EX-enriched samples ($7.2*10^7$ particles/mL).

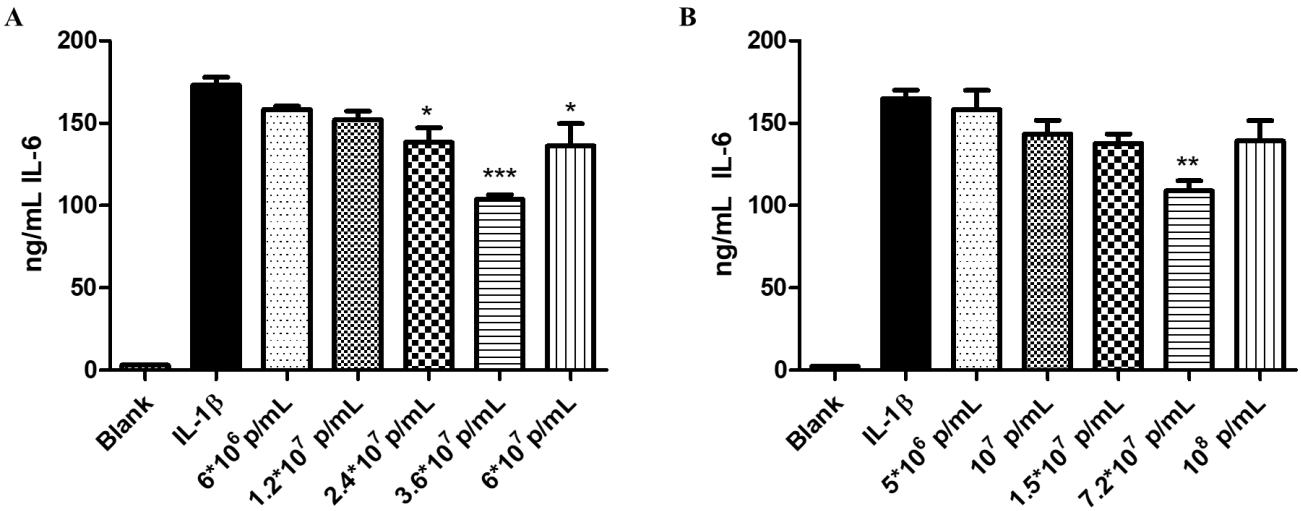

Figure 5. Dose-response curve of MVs **(A)** or EXs **(B)** on the liberation of IL-6 by OA chondrocytes stimulated with IL-1β (10 ng/mL). *p<0.05; **p<0.01; ***p<0.001 respect to cells stimulated with IL-1β.

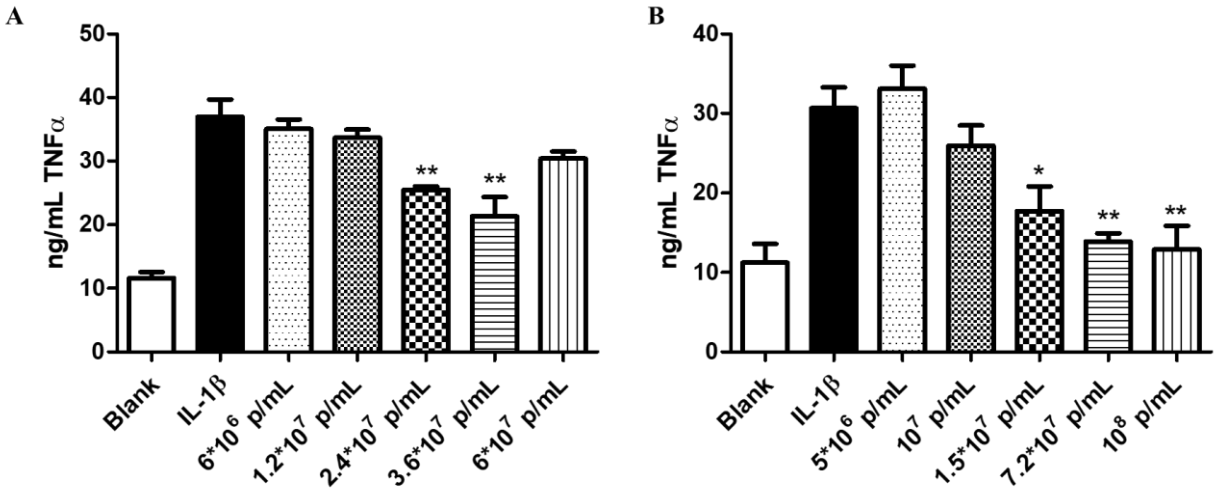

Figure 6. Dose-response curve of MVs **(A)** or EXs **(B)** on the liberation of TNFα by OA chondrocytes stimulated with IL-1β (10 ng/mL). *p<0.05; **p<0.01 respect to cells stimulated with IL-1β.

7. MVs and EXs did not act synergistically

Finally, we assessed whether the combination of both MVs and EXs at their optimal concentration had a synergistic effect when treating OA chondrocytes incubated with IL-1β. As shown in figure 7, the combination of both did not improve the single effect of any of the two. This indicate that the mechanisms of action regulating EV-dependent effects may be specific for each kind of vesicle.

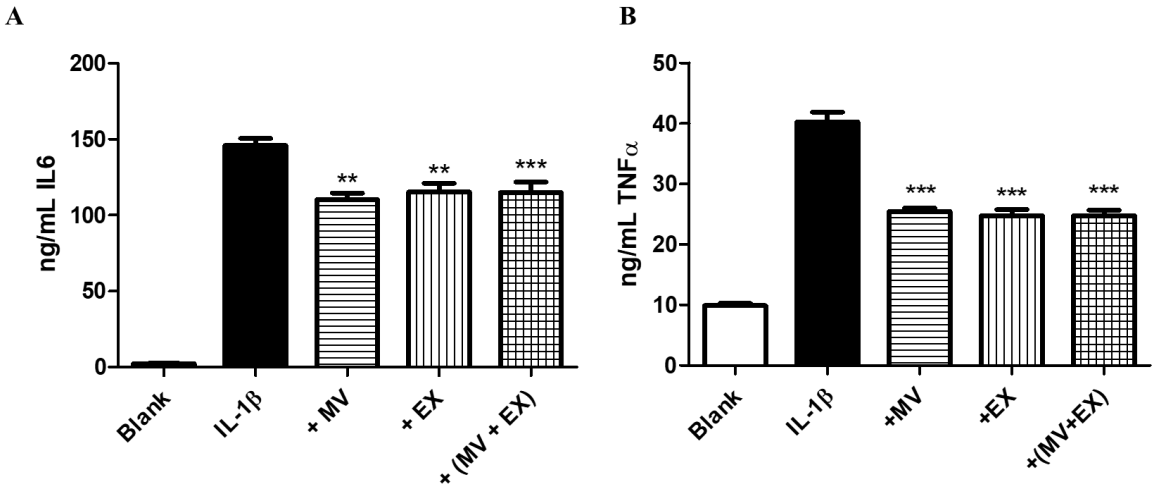

Figure 7. Liberation of IL-6 **(A)** and TNFα **(B)** by OA chondrocytes stimulated with IL-1β after treatment with MVs ($3.6*10^7$ p/mL), EXs ($7.2*10^7$ p/mL) or both. **p<0.01; ***p<0.001 respect to cells stimulated with IL-1β.

Conclusion

Measuring the concentration and size distribution of EVs can be challenging, because of their polydisperse nature. Filtration with 0.1-µm or 0.22-µm filters has been previously used to remove larger particles and multiprotein structures, but has the negative side effect of sample loss and size distribution distortions, so much so that there is a clear need for standardized methodologies for EV concentration measurements (Vogel, Coumans et al. 2016).

Here we have compared three common methods to isolate EVs such as differential centrifugation, SEC and single-step precipitation. Of them, differential centrifugation was our final choice due to its higher yield when processing large volumes, and to its ability to separate different enriched EV subpopulations. Right handling and freezing is of critical importance, especially when dealing with EVs for therapeutic purposes. We have determined the appropriate concentration of EVs from dose-response curves in order to study the effects of these vesicles on OA chondrocytes. Further studies will help uncovering the mechanisms by which EVs inhibit inflammation in chronic inflammatory conditions.

ANEXO II

D. Fernando A. Verdú Pascual, Profesor Titular de Medicina Legal y Forense, y Secretario del Comité Ético de Investigación en Humanos de la Comisión de Ética en Investigación Experimental de la Universitat de València,

CERTIFICA:

Que el Comité Ético de Investigación en Humanos, en la reunión celebrada el día 10 de febrero de 2014, una vez estudiado el proyecto de investigación titulado:

"Mecanismos celulares reguladores de la respuesta inflamatoria en patologías articulares crónicas", número de procedimiento H1389967869063 ,

cuya investigadora responsable es Dña. Mª José Alcaraz Tormo, ha acordado informar favorablemente el mismo dado que se respetan los principios fundamentales establecidos en la Declaración de Helsinki, en el Convenio del Consejo de Europa relativo a los derechos humanos y cumple los requisitos establecidos en la legislación española en el ámbito de la investigación biomédica, la protección de datos de carácter personal y la bioética.

Y para que conste, se firma el presente certificado en Valencia, a once de febrero de dos mil catorce.

FERNANDO
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11:26:13 +01'00'

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Instituto de Investigación Sanitaria La Fe

Dirección General, José Vicente Castell

Instituto de Investigación Sanitaria La Fe
SALIDA
- 9 JUN. 2014
Nº 0376

Mª José Alcaraz Tormo
Universidad de Valencia

Asunto: Autorización Inicio Estudio.

Valencia, 4 de Junio de 2014 .

Adjunto le remito copia de los Informes Científico y Ético de Investigación, en el que se acuerda informar **favorablemente**.

A la vista de los dictámenes emitidos, el Proyecto de Investigación titulado: "MECANISMOS CELULARES REGULADORES DE LA RESPUESTA INFLAMATORIA EN PATOLOGÍAS ARTICULARES CRÓNICAS", puede iniciarse y llevarse a cabo.

Atentamente,

Instituto de Investigación Sanitaria La Fe
CIF: G-97067557

José Vicente Castell Ripoll
Director General

Valencia a 4 de Junio de 2014.

D. José Vte. Castell Ripoll, Presidente de la Comisión de Investigación del Hospital Universitario La Fe de Valencia,

INFORMA:

Que el Proyecto de Investigación titulado: "MECANISMOS CELULARES REGULADORES DE LA RESPUESTA INFLAMATORIA EN PATOLOGÍAS ARTICULARES CRÓNICAS" que presenta el/la Dr./Dra. **Mª José Alcaraz Tormo** de la Universidad Valencia, contiene elementos objetivos suficientes en cuanto a la Hipótesis, Planteamientos y Plan de Trabajo que, a juicio de esta Comisión, permiten pronunciarse **positivamente** en cuanto a su viabilidad.

P.O.

GENERALITAT VALENCIANA · CONSELLERIA DE SANITAT ·
HOSPITAL "LA FE" COMISIÓN DE INVESTIGACIÓN

José Vicente Castell Ripoll
Presidente de la Comisión de Investigación

DICTAMEN DEL COMITÉ ÉTICO DE INVESTIGACIÓN BIOMÉDICA

Don Serafín Rodríguez Capellán, Secretario del Comité Ético de Investigación Biomédica del Hospital Universitario y Politécnico La Fe,

C E R T I F I C A

Que este Comité ha evaluado en su sesión de fecha **6 de mayo de 2014**, el Proyecto de Investigación titulado **"MECANISMOS CELULARES REGULADORES DE LA RESPUESTA INFLAMATORIA EN PATOLOGÍAS ARTICULARES CRÓNICAS."**, con nº de registro **2014/0054**.

Que dicho proyecto se ajusta a las normativas éticas sobre investigación biomédica con sujetos humanos y es viable en cuanto al planteamiento científico, objetivos, material y métodos, etc, descritos en la solicitud, así como la Hoja de Información al Paciente y el Consentimiento Informado.

En consecuencia este Comité acuerda emitir **INFORME FAVORABLE** de dicho Proyecto de Investigación que será realizado en el Hospital Universitario y Politécnico La Fe por el/la **Dr. / Dra. Mª JOSE ALCARAZ TORMO** del servicio de **UNIVERSIDAD DE VALENCIA** como Investigador Principal.

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Vicepresidente:
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Secretario:
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Dra. BEGOÑA POLO MIQUEL ((Gastroenterología Pediátrica)
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Dr. RAFAEL BOTELLA ESTRADA (Dermatología)

Lo que firmo en Valencia, a 6 de mayo de 2014

GENERALITAT VALENCIANA - CONSELLERIA DE SANITAT - AGÈNCIA VALENCIANA DE SALUT
HOSPITAL "LA FE" COMITÉ ÉTICO DE INVESTIGACIÓN BIOMÉDICA

Fdo.: Don Serafín Rodríguez Capellán
Secretario del Comité Ético de Investigación Biomédica

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;47:11-25
DOI: 10.1159/000489739
Published online: May 15, 2018
Accepted: March 29, 2018

Original Paper

Microvesicles from Human Adipose Tissue-Derived Mesenchymal Stem Cells as a New Protective Strategy in Osteoarthritic Chondrocytes

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Key Words
Extracellular vesicles • Adipose tissue-derived mesenchymal stem cells • Chondrocyte • Inflammation • Osteoarthritis

Abstract
Background/Aims: Chronic inflammation contributes to cartilage degeneration during the progression of osteoarthritis (OA). Adipose tissue-derived mesenchymal stem cells (AD-MSC) show great potential to treat inflammatory and degradative processes in OA and have demonstrated paracrine effects in chondrocytes. In the present work, we have isolated and characterized the extracellular vesicles from human AD-MSC to investigate their role in the chondroprotective actions of these cells. ***Methods:*** AD-MSC were isolated by collagenase treatment from adipose tissue from healthy individuals subjected to abdominal lipectomy surgery. Microvesicles and exosomes were obtained from conditioned medium by filtration and differential centrifugation. Chondrocytes from OA patients were used in primary culture and stimulated with 10 ng/ml interleukin(IL)-1β in the presence or absence of AD-MSC microvesicles, exosomes or conditioned medium. Protein expression was investigated by ELISA and immunofluorescence, transcription factor-DNA binding by ELISA, gene expression by real-time PCR, prostaglandin E_2 (PGE_2) by radioimmunoassay, and matrix metalloproteinase (MMP) activity and nitric oxide (NO) production by fluorometry. ***Results:*** In OA chondrocytes stimulated with IL-1β, microvesicles and exosomes reduced the production of inflammatory mediators tumor necrosis factor-α, IL-6, PGE_2 and NO. The downregulation of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 would lead to the decreased PGE_2 production while the effect on NO could depend on the reduction of inducible nitric oxide synthase

M. I. Guillén and M. J. Alcaraz contributed equally to this work.

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expression. Treatment of OA chondrocytes with extracellular vesicles also decreased the release of MMP activity and MMP-13 expression whereas the production of the anti-inflammatory cytokine IL-10 and the expression of collagen II were significantly enhanced. The reduction of inflammatory and catabolic mediators could be the consequence of a lower activation of nuclear factor-κB and activator protein-1. The upregulation of annexin A1 specially in MV may contribute to the anti-inflammatory and chondroprotective effects of AD-MSC. ***Conclusions***: Our data support the interest of AD-MSC extracellular vesicles to develop new therapeutic approaches in joint conditions.

Introduction

Chronic production of inflammatory mediators has important implications for chondrocyte metabolism in joint diseases. Pro-inflammatory cytokines contribute to alterations in osteoarthritis (OA) joint metabolism through the production of inflammatory mediators and catabolic enzymes capable of destroying cartilage matrix [1-3], and the downregulation of anti-inflammatory and anabolic genes [4]. OA remains a leading cause of disability in the elderly without an effective treatment. At present, the treatment of OA is targeted to control symptoms although innovative therapeutic approaches such as joint injection of mesenchymal stem cells (MSC) and differentiation into chondrocytes using appropriate scaffolds to regenerate cartilage are being investigated [5].

MSC have opened a new avenue for treating tissue injury and inflammation. In particular, adipose tissue-derived mesenchymal stem cells (AD-MSC) show great therapeutic potential and have demonstrated protective properties in animal models of OA. Therefore, injection of these cells into the knee joint is able to reduce inflammation and cartilage degradation induced by collagenase in mice [6] or by anterior cruciate ligament transection in rabbits [7].

MSC cell therapy has demonstrated beneficial effects despite short-lived survival of the delivered cells suggesting that secreted factors may be the active components. There is evidence that MSC secrete into their microenvironment a number of cytokines and growth factors that regulate intracellular signaling pathways in neighboring cells, promote angiogenesis and recruitment of stem/progenitor cells, or exert trophic and immunomodulatory effects (reviewed in [8]). As a result, treatment of OA chondrocytes or synovial cells with conditioned medium (CM) from bone marrow MSC or AD-MSC in an inflammatory environment can inhibit the production of inflammatory and catabolic agents [9, 10].

It is now recognized that MSC release extracellular vesicles (EV) as vehicles for intercellular communication. In particular, microvesicles (MV) are a heterogeneous population of spherical structures with a diameter of 100–1000 nm which are released by ectocytosis of the plasma membrane [11] while exosomes (EX) are membrane vesicles with a diameter of 40–100 nm, formed by endocytosis, stored intracellularly and secreted when endosomal structures fuse with the plasma membrane [12]. In recent years, there has been significant interest in MSC EV as mediators of regenerative responses with potential therapeutic applications in cardiovascular diseases [13], rheumatic diseases [14], fracture healing [15], neurodegeneration [16] or immunomodulation [17]. In relation with cartilage metabolism, it has been shown recently that EX from HuES9 human embryonic stem cells are able to repair osteochondral defects in rats [18] and EX from miR-140-5p-overexpressing human synovial MSC prevent the development of OA-like changes after surgical destabilization of the rat knee [19].

We have previously reported the anti-inflammatory and protective properties of CM from AD-MSC in OA chondrocytes [20, 21]. Despite these studies, the possible contribution of EV to the observed effects is not known. To address this issue, we have assessed how MV and EX isolated from this CM could affect the metabolism of OA chondrocytes by modulating inflammatory and degradative pathways relevant in joint destruction.

Materials and Methods

Adipose tissue-derived mesenchymal stem cells

AD-MSC were obtained from the adipose tissue of 10 non-obese donors (4 men and 7 women, aged 53.8±7.4 years, mean±SEM) who had undergone abdominoplasty. The experimental design was approved by the Institutional Ethical Committees (University of Valencia and La Fe Polytechnic University Hospital, Valencia, Spain). Samples were obtained from donors after they provided informed consent according to the Helsinki Declaration of 1975, as revised in 2013.

Adipose tissue samples were washed with phosphate-buffered saline (PBS), minced, digested at 37ºC for 1 h with 2% of type I collagenase (Gibco, Life Technologies, Madrid, Spain) and filtered through a 100 µm cell strainer (BD Biosciences, Bedford, MA, USA). Then, cells were washed with DMEM/HAM F12 (Sigma-Aldrich, St. Louis, MO, USA) containing penicillin and streptomycin (1%), seeded onto tissue culture flasks ($1–2×10^6$ cells/ml) in DMEM/HAM F12 medium with penicillin and streptomycin (1%) supplemented with 15% EV-free human serum, and incubated at 37ºC in a 5% CO_2 atmosphere. Human serum was obtained from whole-blood donations of AB-blood-group-typed donors according to the criteria of Valencia Transfusion Centre. To eliminate the EV fraction, serum was centrifuged during 18 h at 120, 000×g and 4ºC using a SW-28 swinging-bucket rotor (Beckman Coulter, Brea, CA, USA). At 24 h, when cells reached semiconfluence, culture plates were washed and the AD-MSC phenotype confirmed by flow cytometry (Flow Cytometer II, BD Biosciences, San Jose, CA, USA) using specific antibodies: anti-CD105-PE, antiCD90PerCP-eFluo 710, anti-CD34APC (eBioscience, Inc., San Diego, CA, USA), and anti-CD45-PE (BD Pharmingen™, BD Biosciences). Cellular viability was determined with propidium iodide.

Immortalized Human Keratinocytes (HaCaT) cell line was kindly provided by Prof. Norbert E. Fusenig (German Cancer Research Institute, Heidelberg, Germany). Cells were cultured in DMEM/HAM F12 with penicillin and streptomycin (1%) supplemented with 15% EV-free human serum, and incubated at 37ºC in a 5% CO_2 atmosphere.

CM was collected from AD-MSC and HaCaT culture cells at passage 0 every 48 h of culture. It was pooled, centrifuged, and stored in sterile conditions at −80º C prior to further use.

Isolation of EV

EV were obtained from the CM of AD-MSC and HaCaT cells using a filtration/centrifugation-based protocol. Cellular debris was eliminated by pelleting with centrifugation at 300×g for 10 min at 4°C. EV were then collected from the supernatant through differential centrifugation steps. Briefly, CM was filtered through 800 nm filter (Merck, Darmstadt, Germany) and centrifuged at 12, 200×g for 20 min at 4°C to pellet MV. Then, supernatants were filtered through 200 nm filter (Merck, Darmstadt, Germany) and centrifuged at 100, 000×g for 90 min at 4°C. The resulting pellets containing EX were washed once with sterile PBS, resuspended in 15 µl of PBS and stored at -80ºC until use.

Tunable resistive pulse sensing (TRPS)

EV preparations were analyzed by TRPS using a qNano instrument (IZON Sciences Ltd., Oxford, UK) [22]. NP100, and NP300 nanopore membranes were used to measure the samples of EX and MV, respectively. At least 500 events/sample were counted. Calibration was performed using calibration beads SKP200 and SKP400, provided by the manufacturer.

Transmission electron microscopy (TEM)

EV preparation for TEM was performed by the Microscopy Service (SCSIE, University of Valencia). Briefly, LR-white resin inclusion was performed fixing EV samples with Karnovsky fixative, inclusion in agar, followed by water washing and dehydration in 30% EtOH, 50% EtOH, 70% EtOH and 96% EtOH. Finally, samples were sequentially incubated for 2 h in 33% LR-white resin in 96% EtOH, 66% LR-white resin in 96% EtOH, 66% LR-white resin in 100% EtOH and 100% LR-white resin in 100% EtOH. Samples were filtered in resin and polymerized at 60ºC for 48 h. Then, ultrathin slices (60 nm) were made with a diamond blade (DIATOME, Hartfield, USA) in eyelet grilles in a UC6 Ultracut (Leica, Wetzlar, Germany) and stained with uranyl acetate 2% for 25 min and lead citrate 3% for another 12 min prior to visualization in Jeol-1010 (JEOL Ltd. Tokyo, Japan) at 60 kV. Images were acquired with a digital camera MegaView III with Olympus Image Analysis Software (Olympus, Tokyo, Japan).

For gold-immunostaining, mouse anti-human CD63 monoclonal and goat anti-mouse IgG H&L (10 nm Gold) polyclonal antibodies from Abcam (Cambridge, MA, USA) were used. EV were fixed with Karnovsky's fixative and then processed in resin as described above. Grids containing the samples were blocked with PBS/0.8% bovine serum albumin (BSA)/0.1% gelatin, and 2 µl of primary antibody in PBS/0.5% BSA were added. Grids were then washed with PBS/0.5% BSA, incubated with the gold-labeled secondary antibody in PBS/0.5% BSA for 30 min, and then washed in 100 µl drops of PBS/0.5% BSA. Control grids incubated with only secondary antibodies were also used. The grids were stained with 2% uranyl acetate and then viewed for TEM using a Jeol JEM1010 microscope at 60 kV and images were acquired with a digital camera MegaView III with Olympus Image Analysis Software.

Flow Cytometry

Annexin V positive MV were determined with the FITC Annexin V Detection Kit I (BD Biosciences). Immediately before incubation, antibody was ultracentrifuged and washed in 0.2 µm filtered-PBS to avoid noise, and all solutions were previously filtered. For labeling, EV were diluted down to 500, 000 particles/ml in labeling buffer with FITC-conjugated annexin V and incubated at room temperature for 1h in dark. After labeling, EV were twice washed and recovered in filtered PBS. EV were then analyzed at a flow rate on a LSR Fortessa X-20 flow cytometer (BD Biosciences) and data registered with the software DIVA 8.0 processed with the software FlowJo (FlowJo LLC, Ashland, OR, USA). The gating window for counting EV and discriminating against background noise was set using forward and side scatter plots for Megamix-Plus FSC fluorescent beads (BioCytex, Marseille, France) of diameters 100 nm, 300 nm, 500 nm and 900 nm, and FITC fluorescent positivity established as compared to unlabeled EV and EV-free annexin V and FITC-antibody solutions.

OA chondrocytes

Knee specimens were obtained from patients diagnosed with advanced OA (27 women and 14 men, aged 65.6±12.0 years, mean±SEM) who had undergone total joint replacement. The experimental design was approved by the Institutional Ethical Committees as indicated above. Cartilage was dissected from the femoral condyles and tibial plateau of the knee joint and diced into small pieces. Human articular chondrocytes were isolated by sequential enzymatic digestion: 1 h with 0.1 mg/ml hyaluronidase (Sigma-Aldrich) followed by 12–15 h with 2 mg/ml type IA collagenase (Sigma-Aldrich) in DMEM/HAM F12 containing penicillin and streptomycin (1%) at 37ºC in 5% CO_2 atmosphere. The digested tissue was filtered through a 70 µm nylon mesh (BD Biosciences), washed, and centrifuged. Cell viability was greater than 95% according to the Trypan blue exclusion test. All experiments were performed with chondrocyte primary cultures at semiconfluence (270×10^3 cells/well in 6-well plates or 1.5×10^6 cells in 3.5 cm plates). Chondrocytes were maintained with 5% CO_2 at 37ºC in DMEM/HAM F12 containing penicillin and streptomycin (1%), supplemented with 10% fetal bovine serum (Sigma-Aldrich).

To perform the experiments, chondrocytes and explants were incubated for different times in DMEM/HAM F12 containing penicillin and streptomycin (1%) supplemented with 15% EV-free human serum and stimulated with interleukin(IL)-1β (10 ng/ml) in the presence or absence of AD-MSC- or HaCaT MV ($3.6x10^7$ particles/ml), EX ($7.2x10^7$ particles/ml) or CM (0.4 ml for 24-well plates, 1 ml for 6-well plates or 1.5 ml for 3.5 cm plates).

For explant cultures, full-thickness pieces of cartilage were removed from the femoral condyles. Slices measuring ~2 mm in width × 2 mm in length were dissected from the tissue. Explants were transferred to 24-well plates (10 explants/well) containing DMEM/HAM F12 medium supplemented with penicillin and streptomycin (1%), and 10% fetal bovine serum, and they were incubated in a humidified 5% CO_2 incubator at 37°C for 2 days before assays to allow them to stabilize.

MTT Assay

The mitochondrial dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) to formazan was assayed in OA chondrocytes stimulated with IL-1β (10 ng/ml) and treated with MV ($3.6x10^7$ particles/ml), EX ($7.2x10^7$ particles/ml) or CM (0.4 ml) in 24-well plates for 24 h. Cells were then incubated with MTT (200 µg/ml) for 2 h. Medium was removed and cells were solubilized in dimethyl sulfoxide (100 µl) to quantitate formazan at 550 nm using a Victor3 microplate reader (PerkinElmer España, Madrid, Spain).

Proteomic analysis of EV by mass spectrometry

Proteomic characterization of EV samples was performed by the Proteomics Service of the Servei Central de Suport a la Investigació Experimental (Universitat de València). Briefly, 10 µg per EV sample were digested with Triton X-100 and separated by 1-D SDS PAGE. Peptides were generated by a trypsin digestion, extracted, and examined by LC using a NanoLC Ultra 1-D plus Exsigent (Eksigent Technologies, Dublin, CA, USA) which was directly connected to an AB SCIEX TripleTOF 5600 mass spectrometer (AB SCIEX, Framingham, MA, USA) in direct injection mode. After LC-MS/MS, the SCIEX.wiff data-files were processed using ProteinPilot v5.0 search engine (AB SCIEX). The resulting ProteinPilot group file was loaded into PeakView® (v2.1, AB SCIEX) and peaks were extracted with a peptide confidence threshold of 99% confidence (Unused Score ≥ 1.3) and a false discovery rate (FDR) lower than 1%. For this, the MS/MS spectra of the assigned peptides were extracted by ProteinPilot, and only the proteins that fulfilled the following criteria were validated: (1) peptide mass tolerance lower than 10 ppm, (2) 99% of confidence level in peptide identification, and (3) complete b/y ions series found in the MS/MS spectrum. The identified proteins were quantified using PeakView® from normalized label-free quantification (LFQ) intensity data. The quantitative data obtained by PeakView® were analyzed using MarkerView® (v1.2, AB SCIEX). First, areas were normalized by total areas summa. Principal Component Analysis (PCA) was performed to evaluate the discriminative ability of proteins in different EV fractions. Bioinformatics analysis of identified and validated SP-proteins was manually performed using the comprehensive bioinformatics tool for functional annotation UniProt KB database (www.uniprot.org) in combination with PANTHER (www.pantherdb.org) and FunRich (http://www.funrich.org). Data are available via ProteomeXchange with identifier PXD009077 and 10.6019/PXD009077.

ELISA

Chondrocytes were stimulated with IL-1β (10 ng/ml) in presence or absence of MV ($3.6x10^7$ particles/ml), EX ($7.2x10^7$ particles/ml) or CM (1 ml) for 24 h, or 1h for transcription factors detection. Supernatants were centrifuged and stored at -80ºC until analysis. Tumor necrosis factor-α (TNFα), IL-6, and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA) kits from eBioscience (San Diego, CA, USA) with a sensitivity of 4.0 pg/ml for TNFα and IL-6, and 2.0 pg/ml for IL-10. Nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) binding to DNA was quantified by ELISA in nuclear extracts using the Nuclear Extract Kit Active Motif for nuclei extraction followed by TransAM p65 NF-κB and TransAM c-Jun Activation Assay kits (Active Motif Europe, Rixensart, Belgium), according to the manufacturer's recommendations.

Determination of MMP activity, NO and PGE_2

Chondrocytes were stimulated as indicated above and supernatants were harvested and centrifuged. For matrix metalloproteinase (MMP) activity determination, supernatants were incubated with p-aminophenylmercuric acetate for 12 h at 37ºC to activate MMPs. Then, supernatants were transferred to a 96-well plate. After addition of the 5- FAM peptide substrate (AnaSpec Inc., San Jose, CA, USA), fluorescence was measured at 490 nm (excitation)/520 nm (emission) in a Victor3 microplate reader (PerkinElmer España). Prostaglandin E_2 (PGE_2) was quantitated in supernatants by radioimmunoassay [23] and nitric oxide (NO) production was assessed by fluorometric determination of nitrite levels [24] using a Victor3 microplate reader (PerkinElmer España).

Real-time PCR

Total RNA was extracted from OA chondrocytes using the TriPure reagent (Roche Life Science, Barcelona, Spain) according to the manufacturer's instructions. Reverse transcription was accomplished on 1 µg of total RNA using random primers and Transcriptor First Strand cDNA Synthesis Kit (Roche Life Science). PCR assays were performed in duplicate on an iCycler Real-Time PCR Detection System using SYBR Green PCR Master Mix (Bio-Rad Laboratories, Richmond, CA, USA). Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany) [20]. For each sample, differences in threshold cycle (ΔCt) values were calculated by correcting the Ct of the gene of interest to the Ct of the reference gene β-actin. Relative gene expression was expressed as $2^{-\Delta\Delta Ct}$ with respect to non-stimulated cells.

Immunofluorescence

Chondrocytes were seeded at $20x10^3$ cells/well in Lab-tek chambers (Thermo Scientific, Rochester, NY, USA) and stimulated with IL-1β (10 ng/ml) in the presence or absence of MV ($3.6x10^7$ particles/ml), EX ($7.2x10^7$ particles/ml) or CM (0.2 ml) for 24h (annexin A1) or 5 days (collagen II). Cells were fixed with 4%

formaldehyde in PBS for 30 min at 4ºC, blocked with 1% BSA in PBS for 20 min at room temperature and incubated with rabbit anti-human type II collagen polyclonal antibody (Chemicon/Millipore, Schwalbach, Germany) or MaxPab rabbit anti-annexin A1 polyclonal antibody (Abnova, New Taipei, Taiwan) followed by incubation with goat anti-rabbit IgG-FITC (R&D Biosystems, Abingdon, UK). Slides were mounted in Prolong Gold antifade reagent with DAPI (Molecular Probes, Invitrogen, Life Technologies) and examined under a confocal microscope (Olympus FV1000, Tokyo, Japan). Collagen II- or annexin A1-positive cells were observed in 6 microscopic fields of each well. Fluorescence density was quantified using ImageJ software (National Institutes of Health, USA).

Annexin A1 blocking

MV were incubated with MaxPab rabbit anti-ANXA1 polyclonal antibody (Abnova, New Taipei, Taiwan) at 20 µg/ml for 1 h at 4ºC, then washed with PBS and pelleted at 12.600×g. Chondrocytes were stimulated with IL-1β (10 ng/ml) in presence or absence of MV ($3.6x10^7$ particles/ml) previously treated with anti-annexin A1 antibody or MV control for 24 h. IL-6 was determined in supernatants by ELISA and collagen type-II in chondrocytes by immunofluorescence as indicated above.

Statistical analysis

Data are expressed as mean and standard error of the mean (mean ± SEM). Data were analyzed by one-way analysis of variance (ANOVA) followed by the Sidak's post test using the GraphPad Prism 7.0 software (Graph Pad Software, La Jolla, CA, USA). A *P* value of less than 0.05 was considered statistically significant.

Results

Characterization of EV and effect on cell viability

MV and EX fractions were isolated from AD-MSC CM as indicated in Materials and methods. TRPS analysis indicated a mean concentration of MV and EX of $8.0x10^9$ and $3.8x10^{10}$ particles/ml, respectively. In addition, we isolated MV and EX from HaCaT cells as a negative control in functional studies. The MV fraction had an average size of 279±94 nm and a concentration of $6.5x10^{10}$ particles/ml while the EX fraction had an average size of 104±19 nm and a concentration of $1.1x10^{12}$ particles/ml.

Representative TEM images of MV and EX from AD-MSC with estimated size are shown in Fig. 1A. Immunostaining with gold-labeled anti-CD63 antibodies was performed and we observed the presence of CD63 labeling in EX (Fig. 1B). In addition, 56.7±16.4 % (n=3) of annexin V positive MV were detected by flow cytometry. To assess the effects of EV on OA chondrocytes, we selected the concentrations of $3.6x10^7$ particles/ml for MV and $7.2x10^7$ particles/ml for EX which are in the range of concentrations present in CM used in the same experiments. We confirmed that MV and EX at these concentrations do not affect cell viability by the MTT method (Fig. 1C).

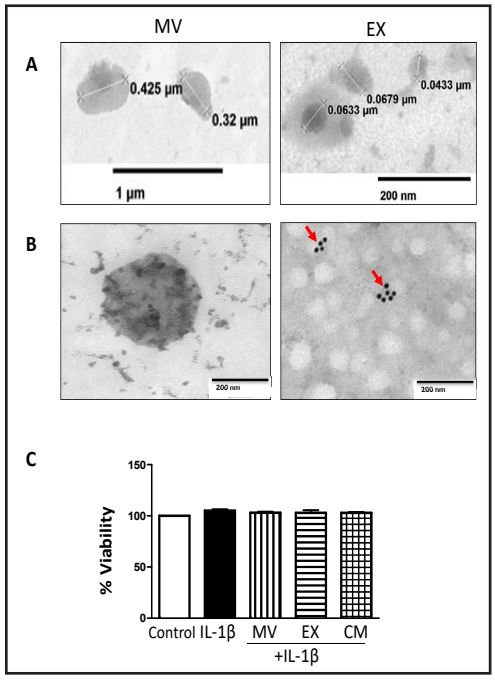

Fig. 1. Characterization of MV and EX isolated from CM from AD-MSC and effect on cell viability. Representative transmission electron microscopy images of MV and EX with estimated size (A). Immunostaining with gold-labeled anti-CD63 antibodies (B). Red arrows show positive staining. C: Viability (%) of OA chondrocytes in the presence of IL-1β and AD-MSC EV, determined by the MTT method. Results are expressed as mean±SEM from 3 separate experiments with cells from separate donors.

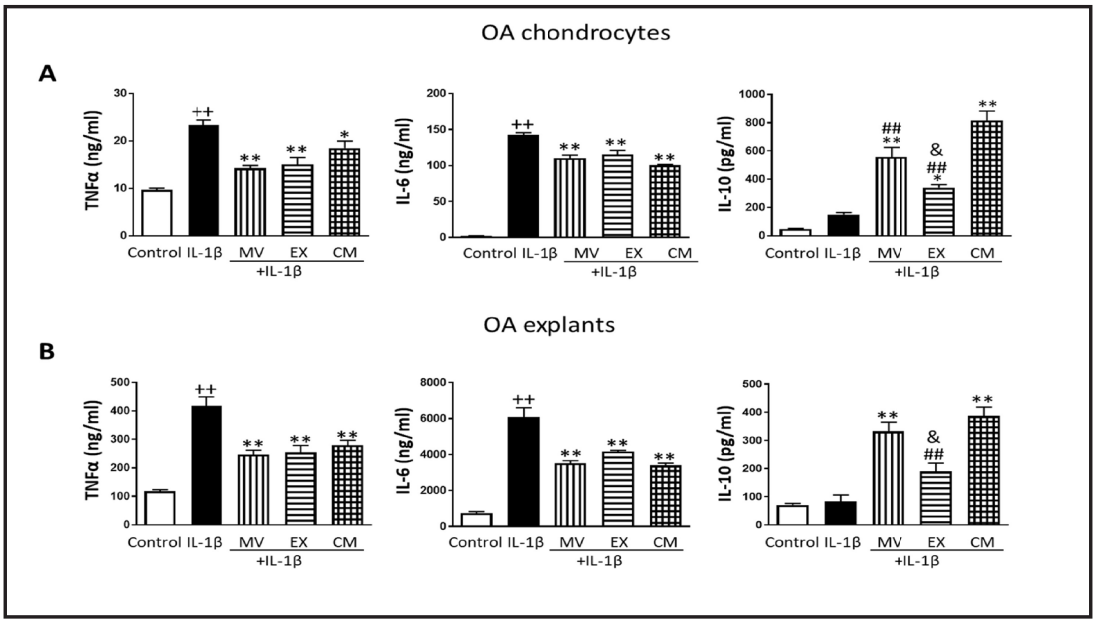

Fig. 2. Effects of EV and CM from AD-MSC on cytokine release by OA chondrocytes (A) and explants (B). IL-6, TNFα and IL-10 were measured by ELISA in culture supernatants. Cultures were treated with IL-1β alone or in combination with EV or CM from AD-MSC for 24 h. Results are expressed as mean±SEM from 3 separate experiments with cells from separate donors. $^{++}$P<0.01 compared to control (non-stimulated cells); *P<0.05, **P<0.01 compared to IL-1β; $^{\#\#}$ P<0.01 compared to CM; $^{\&}$P<0.05 compared to MV.

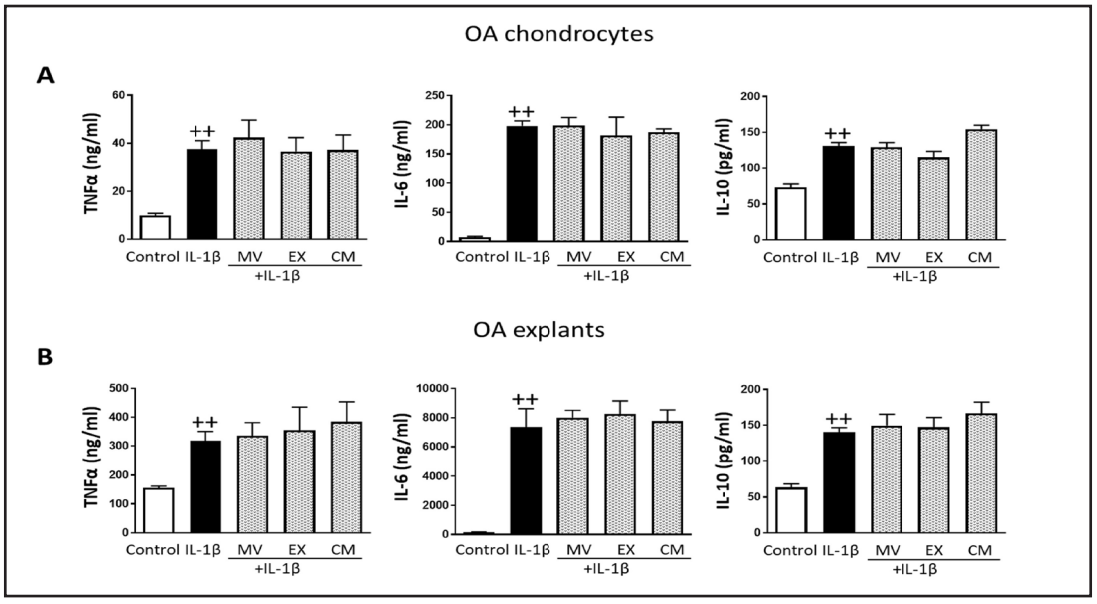

Fig. 3. Effects of EV and CM from HaCaT cells on cytokine release by OA chondrocytes (A) and explants (B). IL-6, TNFα and IL-10 were measured by ELISA in culture supernatants. Cultures were treated with IL-1β alone or in combination with EV or CM from HaCaT cells for 24 h. Results are expressed as mean±SEM from 3 separate experiments with cells from separate donors. $^{++}$P<0.01 compared to control (non-stimulated cells).

Effects on cytokines

To study how the production of relevant inflammatory and catabolic mediators was modulated by AD-MSC-derived EV, OA chondrocytes were incubated with IL-1β in the

presence or absence of MV, EX or CM. Stimulation of OA chondrocytes with IL-1β for 24 h increased the release of the pro-inflammatory cytokines IL-6 and TNFα into the culture medium compared with control non-stimulated cells (Fig. 2A). Treatment with MV, EX or CM significantly reduced the levels of both pro-inflammatory cytokines. In contrast, the release of the anti-inflammatory cytokine IL-10 was significantly enhanced when cells were treated with MV, EX or CM compared with cells treated with IL-1β alone. The highest effect was exhibited by CM followed by MV. In addition, we determined the effects of AD-MSC EV on cytokine production by OA explants which represent a more physiological setting for chondrocytes. As shown in Fig. 2B, the behavior of MV, EX and CM in OA explants was very similar to that observed in OA chondrocytes. In order to determine if these effects of MV and EX are specific to EV from AD-MSC, we performed the same experiments in OA chondrocytes and OA explants but using EV and CM from HaCaT cells instead of AD-MSC. Fig. 3A and B shows that MV, EX and CM from HaCaT cells were completely ineffective on cytokine production by OA chondrocytes or explants.

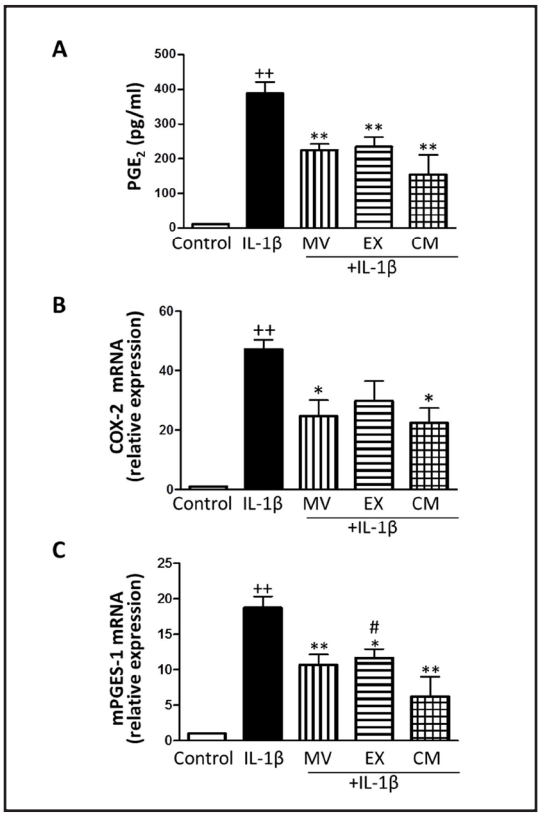

Fig. 4. PGE_2 levels and COX-2 and mPGES-1 gene expression in OA chondrocytes. A: PGE_2 levels were measured by radioimmunoassay in cell culture supernatants. B: COX-2 and C: mPGES-1 mRNA expression was determined by real-time PCR as indicated in materials and methods. Cultures were treated with IL-1β alone or in combination with EV or CM from AD-MSC for 24 h (mean±SEM from 5 separate experiments with cells from separate donors). $^{++}P<0.01$ compared to control (non-stimulated cells); $^{*}P<0.05$, $^{**}P<0.01$ compared to IL-1β; $^{\#}P<0.05$ compared to CM.

Effects on PGE_2 production and COX-2 and mPGES-1 expression

The levels of PGE_2 released into the culture medium of OA chondrocytes were determined to assess if this eicosanoid could be regulated by EV. As shown in Fig. 4A, PGE_2 levels in IL-1β-stimulated cells were significantly decreased by all treatments. IL-1β upregulates cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) which act in a coordinated manner to synthesize high levels of PGE_2 during inflammatory responses [25]. We determined the mRNA expression of COX-2 (Fig. 4B) and mPGES-1 (Fig. 4C) in OA chondrocytes which was reduced by treatment with EV or CM.

Effects on NO production and iNOS expression

IL-1β stimulation of OA chondrocytes results in upregulation of inducible nitric oxide synthase (iNOS) and NO production which was estimated by the levels of nitrite present in the culture medium (Fig. 5A). We observed that MV, EX and CM significantly decreased the levels of nitrite in the medium and the mRNA expression of iNOS in OA chondrocytes compared with cells treated with IL-1β alone.

Effects on MMPs

In order to evaluate the effects of treatments on total MMP activity, a fluorometric assay was performed as indicated in Materials and methods. Fig. 5B shows that OA chondrocyte

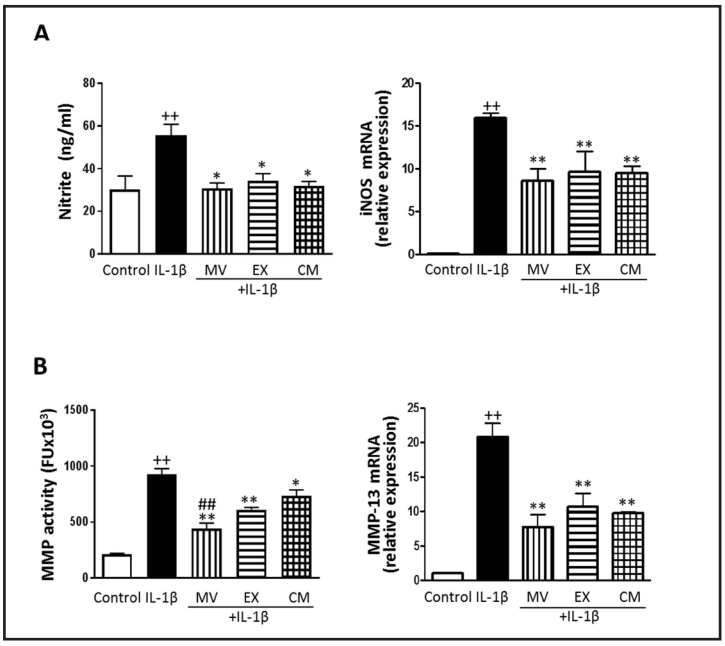

Fig. 5. NO production and iNOS gene expression (A), MMP activity and MMP-13 gene expression (B) in OA chondrocytes. Nitrite levels and MMP activity were measured by fluorometry in cell culture supernatants. iNOS and MMP-13 mRNA expression was determined by real-time PCR as indicated in materials and methods. Cultures were treated with IL-1β alone or in combination with EV or CM from AD-MSC for 24 h (mean±SEM from 4 (A) or 3 (B) separate experiments with cells from separate donors). FU, fluorescence units. $^{++}$P<0.01 compared to control (non-stimulated cells); *P<0.05, **P<0.01 compared to IL-1β; $^{\#\#}$ P<0.01 compared to CM.

stimulation with IL-1β resulted in increased MMP activity in the culture medium. All treatments significantly reduced this activity and MV exerted a stronger effect compared with CM. MMP-13 (collagenase 3) has been implicated in the early phase of chondrocyte-mediated cartilage collagen breakdown [26]. A consistent induction of MMP-13 gene expression was seen following IL-1β stimulation of OA chondrocytes whereas treatment with MV, EX or CM significantly decreased it.

Effects on collagen II expression

It is known that IL-1β suppress the transcription of the chondrocyte-specific marker collagen type II [4] and induces its degradation [27]. Fig. 6 shows that when IL-1β and EV or CM were present in the culture medium, these treatments significantly relieved the IL-1β-induced suppression of chondrocyte-specific collagen type II expression. Interestingly, MV were significantly more effective than EX or CM leading to a level of collagen II close to that of control chondrocytes (non-stimulated cells).

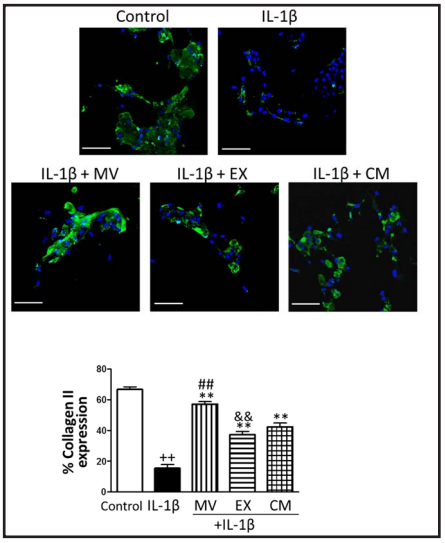

Fig. 6. Collagen II expression in OA chondrocytes. Collagen II protein expression was determined by immunofluorescence. Cultures were treated with IL-1β alone or in combination with EV or CM from AD-MSC for 5 days. Results are expressed as mean±SEM from 4 separate experiments with cells from separate donors. $^{++}$P<0.01 compared to control (non-stimulated cells); **P<0.01 compared to IL-1β; $^{\#\#}$ P<0.01 compared to CM; $^{\&\&}$P<0.01 compared to MV. Bar= 50 μm.

Effects on transcription factors

The transcription factor NF-κB mediates many of the downstream effects of IL-1β activating the transcription of pro-inflammatory and catabolic molecules. We have studied the effects of MV, EX and CM on the binding of p65 to DNA in the nucleus of OA chondrocytes stimulated with IL-1β. There was a marked enhancement of p65-DNA binding by this cytokine (Fig. 7A) which was significantly reduced by MV, CM and to a lesser extent by EX. AP-1 also plays an important role in MMP

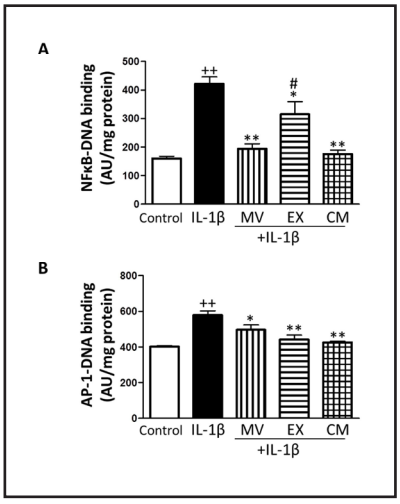

Fig. 7. Activation of transcription factors in OA chondrocytes. P65 NF-κB (A) and c-jun AP-1 (B) binding to DNA was measured by ELISA and expressed as arbitrary units (AU) per mg of protein. Cultures were treated with IL-1β alone or in combination with EV or CM from AD-MSC for 1 h. Results are expressed as mean±SEM from 4 separate experiments with cells from separate donors. $^{++}$P<0.01 compared to control (non-stimulated cells); *P<0.05, **P<0.01 compared to IL-1β; $^{\#}$ P<0.05 compared to CM.

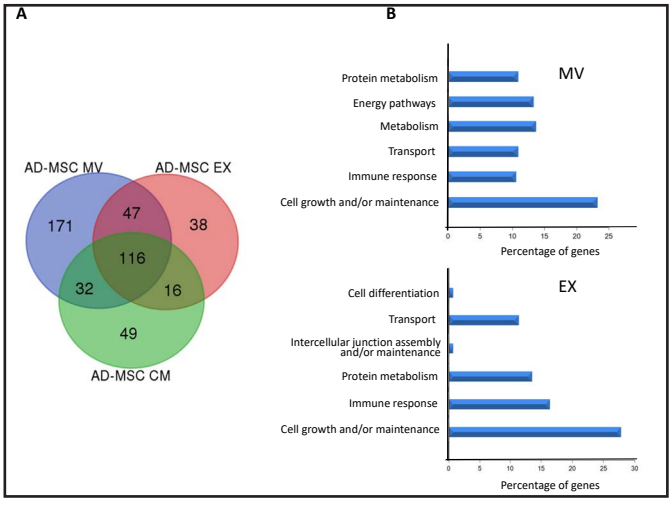

Fig. 8. Proteome characterization of EV from AD-MSC. A: Venn diagram. The number present in the circle represents the total number of identified proteins in particular data sets. B: Most over-represented biological processes (gene ontology terms) present in EV proteomes.

Table 1. Over-represented proteins in AD-MSC EV compared to CM

Identified protein	Uniprot ID	Main function
MV		
Annexin A1	P04083	Immunomodulation
Apolipoprotein H	P02749	Negtive substrate binding
Butyryl-Cholinesterase	P06276	Esterase activity
CD81	P60033	Endocytic trafficking
Dermokine	Q6E0U4	Keratinocyte differentiation
Fermitin family homolog 3	Q86UX7	Integrin activation
Integrin β1	P05556	Cell-matrix collagen binding
Peroxiredoxin 6	P30041	Peroxidase/Phospholipase A_2
Phosphoglycerate mutase 1	P18669	Canonical glycolysis
Junction Plakoglobin	P14923	α-catenin binding
Rab GDP dissociation inhibitor	P50395	Rab regulation
Tropomyosin 1	P09493	Actin binding
Tropomyosin 3	P06753	Actin binding
α-Actinin 4	O43707	Actin binding
α-Enolase	P06733	Canonical glycolysis
EX		
Carboxypeptidase N	P15169	Inflammatory peptides degradation
HSP70	P11142	Molecular chaperone
Pregnancy-zone protein	F5GXY0	Endopeptidase inhibitor

transcription induced by cytokines [1]. DNA binding of c-Jun AP-1 was significantly activated by IL-1β (Fig. 7B) while treatment with MV, EX or CM significantly decreased this process.

Proteomic analysis of AD-MSC EV

We performed a proteomic analysis of AD-MSC EV in order to identify their protein content and possible active components. 365 proteins were found in MV preparations, 217 in EX and 213 in CM. Fig. 8A shows the Venn diagram indicating that 116 proteins were identified in MV, EX and CM. MV had a higher number of unshared proteins compared with EX (171 vs. 38). These proteins are involved in different cellular process, mainly cellular growth and/or maintenance, immune response, protein metabolism and transport (Fig. 8B). When compared with CM proteome, 42 unique proteins were identified in MV with a *P* value <0.01. Of them, 15 (Table 1) were over-represented with a fold-change of at least 2.5. Among EX proteins, 28 were found significantly different from the CM proteome, but only 3 were over-represented with a fold change of at least 2.5.

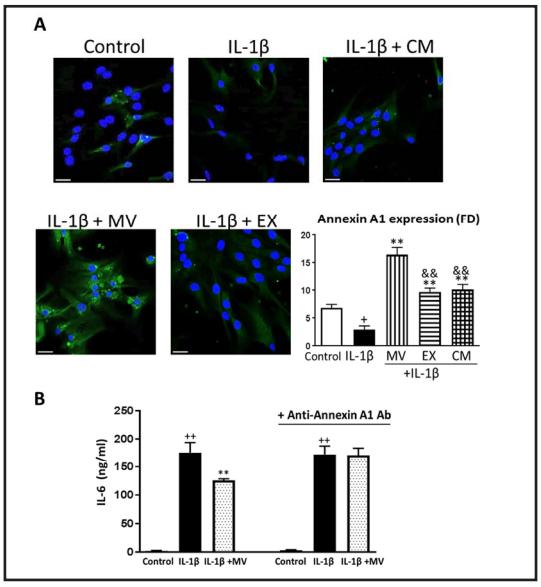

Fig. 9. Annexin A1 expression (A) and IL-6 production after annexin A1 blockade (B) in OA chondrocytes. A: Protein expression was determined by immunofluorescence. Cultures were treated with IL-1β alone or in combination with EV or CM from AD-MSC for 24 h. Results are expressed as mean±SEM from 4 separate experiments with cells from separate donors. FD: fluorescence density. $^{+}$P<0.05 compared to control (non-stimulated cells); **P<0.01 compared to IL-1β. $^{\&\&}$P<0.01 compared to MV. Bar= 30 μm. B: IL-6 was measured by ELISA in cell culture supernatants. Cultures were treated with IL-1β alone or in combination with MV from AD-MSC after annexin A1 blockade with a specific antibody or without blockade. Results are expressed as mean±SEM from 3 separate experiments with cells from separate donors. $^{++}$P<0.01 compared to control (non-stimulated cells); **P<0.01 compared to IL-1β.

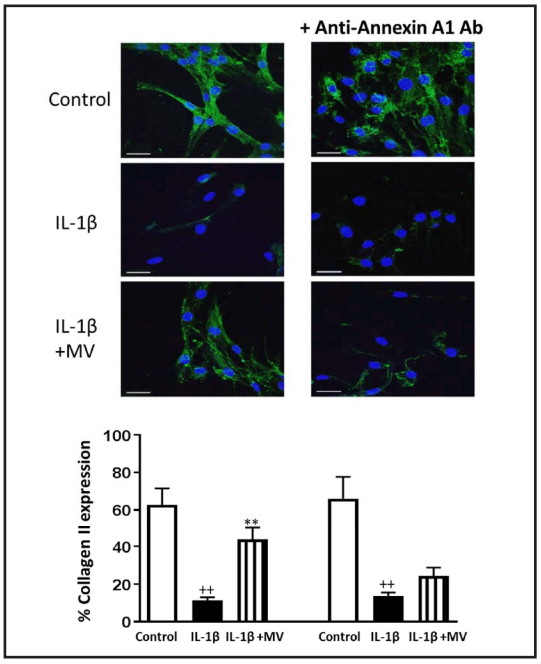

Fig. 10. Collagen II expression after annexin A1 blockade. Collagen II protein expression was determined by immunofluorescence. Cultures were treated with IL-1β alone or in combination with MV from AD-MSC after annexin A1 blockade with a specific antibody or without blockade. Results are expressed as mean±SEM from 3 separate experiments with cells from separate donors. $^{++}$P<0.01 compared to control (non-stimulated cells); **P<0.01 compared to IL-1β. Bar= 30 μm.

Effects on annexin A1

We have studied CM and EV effects on the expression of annexin A1. This protein is over-represented in MV (Table 1) and has demonstrated immunomodulatory and anti-inflammatory properties in different systems (reviewed in [28]). Fig. 9A shows that annexin A1 expression was significantly enhanced by treatment of chondrocytes with all fractions and mainly with MV. To explore the possibility that annexin A1 may contribute to the observed anti-inflammatory and chondroprotective effects, we neutralized this protein in the fraction showing the highest expression using a specific antibody and determined the consequences on the production of the inflammatory cytokine IL-6 and the expression of collagen II in OA chondrocytes in the presence of IL-1β. Annexin A1 blockade significantly reverted the inhibitory effects of MV on the inflammatory cytokine IL-6 (Fig. 9B) and the enhancement of type II collagen (Fig. 10).

Discussion

A wide range of evidence indicates that paracrine effects of MSC are a central mechanism of cell therapy promoting tissue regeneration [29, 30]. In line with this view, we have previously shown that CM from AD-MSC exhibits anti-inflammatory properties in OA chondrocytes [20]. In the present work, we

have characterized the EV present in this CM and assessed their possible contribution to its protective actions.

Synovitis and pro-inflammatory mediators are present in early-stage and late-stage OA (reviewed in [31]) and participate in a positive inflammatory feedback loop in the joint between synoviocytes and chondrocytes likely involving EV [32]. In OA cartilage, IL-1β and TNFα induce a wide range of pro-inflammatory mediators such as cytokines, chemokines, PGE_2, NO and degradative enzymes [31, 33]. Therefore, inflammation is associated to progression of cartilage damage in OA and different mediators synergize to amplify and perpetuate the process. Our results have shown that MV and EX present in CM from AD-MSC exert anti-inflammatory effects similar to CM. In contrast, EV from HaCaT cells were completely ineffective on cytokine production in OA chondrocytes and explants confirming the specificity of the observed effects. The downregulation of the pro-inflammatory cytokines TNFα and IL-6 may have implications for the control of altered chondrocyte metabolism. Interestingly, IL-6 has been involved in OA pathophysiology [34] and increased circulating levels of IL-6 have been associated to radiographic knee OA [35].

MV and EX were also able to counteract the inhibitory effects of IL-1β on the anti-inflammatory cytokine IL-10. MV treatment determined a significant IL-10 enhancement compared with EX, which may be related to its content of annexin A1 [36]. The increased production of IL-10 may then contribute to the anti-inflammatory effects of CM and its EV as this cytokine inhibits the synthesis of pro-inflammatory cytokines [37] and antagonizes their deleterious effects on chondrocyte metabolism [38, 39].

Induction of COX-2 and mPGES-1 and enhanced PGE_2 synthesis in articular chondrocytes lead to anti-anabolic and degradative effects in the joint [25, 40, 41]. Our results indicate that MV and EX are able to control the levels of this eicosanoid through the downregulation of COX-2 and mPGES-1. In addition, MV and EX decreased iNOS induction and NO production, thus preventing the effects of this mediator on the induction and activation of MMPs and the inhibition of extracellular matrix synthesis [42].

The actions of inflammatory mediators in chondrocytes also result in a reduced collagen II expression in OA chondrocytes [27]. Our data indicate that EV from AD-MSC protect OA chondrocytes from the negative effects of IL-1β on collagen II. These findings suggest a role for EV as protective mediators of the differentiated chondrocyte phenotype in inflammatory conditions. Components of extracellular matrix and inflammatory mediators stimulate the degradation of cartilage by inducing different catabolic enzymes. We have shown that MV and to a lesser extent EX, reduced the release of MMP activity. This was accompanied by a significant reduction in gene expression of MMP-13 which plays a key role in collagen II degradation [43]. Taken as a whole, our results suggest that MV may provide better chondroprotection than EX or CM from AD-MSC.

In OA chondrocytes, canonical NF-κB signaling mediates the induction of inflammatory mediators and catabolic mechanisms as well as cellular differentiation changes which favor the onset and perpetuation of disease [44, 45]. A reduction in the activation of this transcription factor by CM and EV could contribute to the observed downregulation of IL-6, TNFα, COX-2, iNOS and MMPs [46]. There is a significant crosstalk of NF-κB with other signaling pathways relevant in OA chondrocytes. In particular, AP-1 cooperates with NF-κB in the induction of MMP-13 and other MMPs [1, 47]. Furthermore, IL-1β suppresses collagen II expression in articular chondrocytes by inducing the activation of AP-1 and subsequent suppression of Sox-9 contributing to the loss of the differentiated chondrocyte phenotype [48]. We have shown that MV and EX decrease the DNA binding activities of AP-1 and NF-κB with an effect of MV similar to CM and higher than EX on the last transcription factor. Therefore, the downregulation of MMP activity and MMP-13 gene expression may be the consequence of a decreased activation of both transcription factors.

Proteomic analysis indicated the presence of unique proteins in MV and EX fractions of AD-MSC CM, and some of them can play a role in the regulation of inflammatory processes and immune responses. In particular, annexin A1 is over-represented in MV and exerts complex anti-inflammatory and pro-resolution effects. In addition to the inhibition of different

inflammatory mediators, annexin A1 exerts suppressive effect on cells of the immune system (reviewed in [49]). EV represent a way of cellular communication and transfer of components which may be exploited for therapeutic purposes. In this regard, annexin A1 is secreted, at least in part, in EV by different cell types such as neutrophils [50] or human bone marrow mesenchymal stem cells [51] and it can be delivered into the recipient cell [50, 52]. Although further studies are needed to assess the possible contribution of other components of AD-MSC EV, our data suggest that annexin A1 may contribute to the anti-inflammatory and chondroprotective effects of these microparticles under inflammatory stress conditions. These findings are in line with the report that neutrophil MV expressing annexin A1 enhanced chondrocyte anabolic properties *in vitro*, and after *in vivo* administration to mice protected against cartilage degradation in a model of inflammatory arthritis [50].

Conclusion

In summary, we have shown that MV and EX present in the CM of AD-MSC modulate chondrocyte metabolism to counteract the effects of IL-1β. Therefore, EV can reproduce the anti-inflammatory properties of CM from AD-MSC in OA chondrocytes. Our findings are consistent with the hypothesis that EV are mediators of AD-MSC chondroprotective actions with a main role for MV. These EV may play important regulatory roles during cell communication and represent a novel strategy to develop potential treatments in joint conditions.

Acknowledgements

This work was supported by grants SAF2013-4874R (MINECO, FEDER) and PROMETEOII/2014/071 (Generalitat Valenciana), Spain.

Disclosure statement

The authors declare to have no conflict of interests.

References

1 Mengshol JA, Vincenti MP, Coon CI, Barchowsky A, Brinckerhoff CE: Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB: differential regulation of collagenase 1 and collagenase 3. Arthritis Rheum 2000;43:801-811.

2 Tetlow LC, Adlam DJ, Woolley DE: Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. Arthritis Rheum 2001;44:585-594.

3 Attur MG, Patel IR, Patel RN, Abramson SB, Amin AR: Autocrine production of IL-1 beta by human osteoarthritis-affected cartilage and differential regulation of endogenous nitric oxide, IL-6, prostaglandin E2, and IL-8. Proc Assoc Am Physicians 1998;110:65-72.

4 Goldring MB, Birkhead J, Sandell LJ, Kimura T, Krane SM: Interleukin 1 suppresses expression of cartilage-specific types II and IX collagens and increases types I and III collagens in human chondrocytes. J Clin Invest 1988;82:2026-2037.

5 Pers YM, Ruiz M, Noël D, Jorgensen C: Mesenchymal stem cells for the management of inflammation in osteoarthritis: state of the art and perspectives. Osteoarthritis Cartilage 2015;23:2027-2035.

6 ter Huurne M, Schelbergen R, Blattes R, Blom A, de Munter W, Grevers LC, Jeanson J, Noël D, Casteilla L, Jorgensen C, van den Berg W, van Lent PLEM: Antiinflammatory and chondroprotective effects of intraarticular injection of adipose-derived stem cells in experimental osteoarthritis. Arthritis Rheum 2012;64:3604-3613.

7 Desando G, Cavallo C, Sartoni F, Martini L, Parrilli A, Veronesi F, Fini M, Giardino R, Facchini A, Grigolo B: Intra-articular delivery of adipose derived stromal cells attenuates osteoarthritis progression in an experimental rabbit model. Arthritis Res Ther 2013;15:R22.

8 Wang Y, Chen X, Cao W, Shi Y: Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. Nat Immunol 2014;15:1009-1016.
9 van Buul GM, Villafuertes E, Bos PK, Waarsing JH, Kops N, Narcisi R, Weinans H, Verhaar JA, Bernsen MR, van Osch GJ: Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explant culture. Osteoarthritis Cartilage 2012;20:1186-1196.
10 Manferdini C, Maumus M, Gabusi E, Piacentini A, Filardo G, Peyrafitte JA, Jorgensen C, Bourin P, Fleury-Cappellesso S, Facchini A, Noel D, Lisignoli G: Adipose-derived mesenchymal stem cells exert antiinflammatory effects on chondrocytes and synoviocytes from osteoarthritis patients through prostaglandin E2. Arthritis Rheum 2013;65:1271-1281.
11 Mause SF, Weber C: Microparticles: protagonists of a novel communication network for intercellular information exchange. Circ Res 2010;107:1047-1057.
12 Colombo M, Raposo G, Théry C: Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol 2014;30:255-289.
13 Lai RC, Chen TS, Lim SK: Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. Regen Med 2011;6:481-492.
14 Maumus M, Jorgensen C, Noel D: Mesenchymal stem cells in regenerative medicine applied to rheumatic diseases: Role of secretome and exosomes. Biochimie 2013;95:2229-2234.
15 Furuta T, Miyaki S, Ishitobi H, Ogura T, Kato Y, Kamei N, Miyado K, Higashi Y, Ochi M: Mesenchymal Stem Cell-Derived Exosomes Promote Fracture Healing in a Mouse Model. Stem Cells Transl Med 2016;5:1620-1630.
16 Sarko DK, McKinney CE: Exosomes: Origins and Therapeutic Potential for Neurodegenerative Disease. Front Neurosci 2017;11:82.
17 Zhang B, Yin Y, Lai RC, Tan SS, Choo AB, Lim SK: Mesenchymal stem cells secrete immunologically active exosomes. Stem Cells Dev 2014;23:1233-1244.
18 Zhang S, Chu WC, Lai RC, Lim SK, Hui JH, Toh WS: Exosomes derived from human embryonic mesenchymal stem cells promote osteochondral regeneration. Osteoarthritis Cartilage 2016;24:2135-2140.
19 Tao SC, Yuan T, Zhang YL, Yin WJ, Guo SC, Zhang CQ: Exosomes derived from miR-140-5p-overexpressing human synovial mesenchymal stem cells enhance cartilage tissue regeneration and prevent osteoarthritis of the knee in a rat model. Theranostics 2017;7:180-195.
20 Platas J, Guillen MI, del Caz MD, Gomar F, Mirabet V, Alcaraz MJ: Conditioned media from adipose tissue-derived mesenchymal stem cells down-regulate degradative mediators induced by interleukin-1â in osteoarthritic chondrocytes. Mediators Inflamm 2013;2013/357014:
21 Platas J, Guillen MI, Perez Del Caz MD, Gomar F, Castejon MA, Mirabet V, Alcaraz MJ: Paracrine effects of human adipose-derived mesenchymal stem cells in inflammatory stress-induced senescence features of osteoarthritic chondrocytes. Aging (Albany NY) 2016;8:1703-1717.
22 Vogel R, Coumans FA, Maltesen RG, Boing AN, Bonnington KE, Broekman ML, Broom MF, Buzas EI, Christiansen G, Hajji N, Kristensen SR, Kuehn MJ, Lund SM, Maas SL, Nieuwland R, Osteikoetxea X, Schnoor R, Scicluna BJ, Shambrook M, de VJ, Mann SI, Hill AF, Pedersen S: A standardized method to determine the concentration of extracellular vesicles using tunable resistive pulse sensing. J Extracell Vesicles 2016;5:31242.
23 Moroney MA, Alcaraz MJ, Forder RA, Carey F, Hoult JRS: Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids. J Pharm Pharmacol 1988;40:787-792.
24 Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG: A fluorometric assay for the measurement of nitrite in biological samples. Anal Biochem 1993;214:11-16.
25 Li X, Afif H, Cheng S, Martel-Pelletier J, Pelletier JP, Ranger P, Fahmi H: Expression and regulation of microsomal prostaglandin E synthase-1 in human osteoarthritic cartilage and chondrocytes. J Rheumatol 2005;32:887-895.
26 Koshy PJ, Lundy CJ, Rowan AD, Porter S, Edwards DR, Hogan A, Clark IM, Cawston TE: The modulation of matrix metalloproteinase and ADAM gene expression in human chondrocytes by interleukin-1 and oncostatin M: a time-course study using real-time quantitative reverse transcription-polymerase chain reaction. Arthritis Rheum 2002;46:961-967.
27 Goldring MB, Otero M, Tsuchimochi K, Ijiri K, Li Y: Defining the roles of inflammatory and anabolic cytokines in cartilage metabolism. Ann Rheum Dis 2008;67 Suppl 3:iii75-iii82.
28 Perretti M, Dalli J: Exploiting the Annexin A1 pathway for the development of novel anti-inflammatory therapeutics. Br J Pharmacol 2009;158:936-946.

29 Ratajczak MZ, Jadczyk T, Pedziwiatr D, Wojakowski W: New advances in stem cell research: practical implications for regenerative medicine. Pol Arch Med Wewn 2014;124:417-426.
30 Hofer HR, Tuan RS: Secreted trophic factors of mesenchymal stem cells support neurovascular and musculoskeletal therapies. Stem Cell Res Ther 2016;7:131.
31 Goldring MB, Otero M: Inflammation in osteoarthritis. Curr Opin Rheumatol 2011;23:471-478.
32 Withrow J, Murphy C, Liu Y, Hunter M, Fulzele S, Hamrick MW: Extracellular vesicles in the pathogenesis of rheumatoid arthritis and osteoarthritis. Arthritis Res Ther 2016;18:286.
33 Chevalier X: Upregulation of enzymatic activity by interleukin-1 in osteoarthritis. Biomed Pharmacother 1997;51:58-62.
34 Goekoop RJ, Kloppenburg M, Kroon HM, Frolich M, Huizinga TW, Westendorp RG, Gussekloo J: Low innate production of interleukin-1beta and interleukin-6 is associated with the absence of osteoarthritis in old age. Osteoarthritis Cartilage 2010;18:942-947.
35 Livshits G, Zhai G, Hart DJ, Kato BS, Wang H, Williams FM, Spector TD: Interleukin-6 is a significant predictor of radiographic knee osteoarthritis: The Chingford Study. Arthritis Rheum 2009;60:2037-2045.
36 Headland SE, Norling LV: The resolution of inflammation: Principles and challenge. Semin Immunol 2015;27:149-160.
37 Fernandes JC, Martel-Pelletier J, Pelletier JP: The role of cytokines in osteoarthritis pathophysiology. Biorheology 2003;39:237-246.
38 Wang Y, Lou S: Direct protective effect of interleukin-10 on articular chondrocytes *in vitro*. Chin Med J (Engl) 2001;114:723-725.
39 John T, Muller RD, Oberholzer A, Zreiqat H, Kohl B, Ertel W, Hostmann A, Tschoeke SK, Schulze-Tanzil G: Interleukin-10 modulates pro-apoptotic effects of TNF-alpha in human articular chondrocytes *in vitro*. Cytokine 2007;40:226-234.
40 Hardy MM, Seibert K, Manning PT, Currie MG, Woerner BM, Edwards D, Koki A, Tripp CS: Cyclooxygenase 2-dependent prostaglandin E2 modulates cartilage proteoglycan degradation in human osteoarthritis explants. Arthritis Rheum 2002;46:1789-1803.
41 Li X, Ellman M, Muddasani P, Wang JH, Cs-Szabo G, Van Wijnen AJ, Im HJ: Prostaglandin E2 and its cognate EP receptors control human adult articular cartilage homeostasis and are linked to the pathophysiology of osteoarthritis. Arthritis Rheum 2009;60:513-523.
42 Lotz M: The role of nitric oxide in articular cartilage damage. Rheum Dis Clin North Am 1999;25:269-282.
43 Goldring MB, Otero M, Plumb DA, Dragomir C, Favero M, El HK, Hashimoto K, Roach HI, Olivotto E, Borzi RM, Marcu KB: Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. Eur Cell Mater 2011;21:202-220.
44 Chockalingam PS, Varadarajan U, Sheldon R, Fortier E, LaVallie ER, Morris EA, Yaworsky PJ, Majumdar MK: Involvement of protein kinase Czeta in interleukin-1beta induction of ADAMTS-4 and type 2 nitric oxide synthase via NF-kappaB signaling in primary human osteoarthritic chondrocytes. Arthritis Rheum 2007;56:4074-4083.
45 Olivotto E, Otero M, Marcu KB, Goldring MB: Pathophysiology of osteoarthritis: canonical NF-kappaB/IKKbeta-dependent and kinase-independent effects of IKKalpha in cartilage degradation and chondrocyte differentiation. RMD Open 2015;1:e000061.
46 Rigoglou S, Papavassiliou AG: The NF-kappaB signaling pathway in osteoarthritis. Int J Biochem Cell Biol 2013;45:2580-2584.
47 Liacini A, Sylvester J, Li WQ, Zafarullah M: Inhibition of interleukin-1-stimulated MAP kinases, activating protein-1 (AP-1) and nuclear factor kappa B (NF-kappa B) transcription factors down-regulates matrix metalloproteinase gene expression in articular chondrocytes. Matrix Biol 2002;21:251-262.
48 Hwang SG, Yu SS, Poo H, Chun JS: c-Jun/activator protein-1 mediates interleukin-1beta-induced dedifferentiation but not cyclooxygenase-2 expression in articular chondrocytes. J Biol Chem 2005;280:29780-29787.
49 D'Acquisto F, Perretti M, Flower RJ: Annexin-A1: a pivotal regulator of the innate and adaptive immune systems. Br J Pharmacol 2008;155:152-169.
50 Headland SE, Jones HR, Norling LV, Kim A, Souza PR, Corsiero E, Gil CD, Nerviani A, Dell'Accio F, Pitzalis C, Oliani SM, Jan LY, Perretti M: Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis. Sci Transl Med 2015;7:315ra190.
51 Kim HS, Choi DY, Yun SJ, Choi SM, Kang JW, Jung JW, Hwang D, Kim KP, Kim DW: Proteomic analysis of microvesicles derived from human mesenchymal stem cells. J Proteome Res 2012;11:839-849.
52 Leoni G, Nusrat A: Annexin A1: shifting the balance towards resolution and repair. Biol Chem 2016;397:971-979.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 7197598, 12 pages
https://doi.org/10.1155/2017/7197598

Research Article

Extracellular Vesicles from Adipose-Derived Mesenchymal Stem Cells Downregulate Senescence Features in Osteoarthritic Osteoblasts

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Received 29 July 2017; Accepted 3 October 2017; Published 5 November 2017

Academic Editor: Silvia Cetrullo

Osteoarthritis (OA) affects all articular tissues leading to pain and disability. The dysregulation of bone metabolism may contribute to the progression of this condition. Adipose-derived mesenchymal stem cells (ASC) are attractive candidates in the search of novel strategies for OA treatment and exert anti-inflammatory and cytoprotective effects on cartilage. Chronic inflammation in OA is a relevant factor in the development of cellular senescence and joint degradation. In this study, we extend our previous observations of ASC paracrine effects to study the influence of conditioned medium and extracellular vesicles from ASC on senescence induced by inflammatory stress in OA osteoblasts. Our results in cells stimulated with interleukin- (IL-) 1β indicate that conditioned medium, microvesicles, and exosomes from ASC downregulate senescence-associated β-galactosidase activity and the accumulation of γH2AX foci. In addition, they reduced the production of inflammatory mediators, with the highest effect on IL-6 and prostaglandin E_2. The control of mitochondrial membrane alterations and oxidative stress may provide a mechanism for the protective effects of ASC in OA osteoblasts. We have also shown that microvesicles and exosomes mediate the paracrine effects of ASC. Our study suggests that correction of abnormal osteoblast metabolism by ASC products may contribute to their protective effects.

1. Introduction

Osteoarthritis (OA) is the most prevalent joint disease and a leading cause of pain and disability in the aging population. OA affects the whole joint leading to cartilage degradation, synovitis, formation of osteophytes, and bone sclerosis. Several studies have demonstrated that bone metabolism is dysregulated in OA and may contribute to the onset and/or progression of this condition [1, 2]. Therefore, the modification of the abnormal metabolism of bone cells may lead to novel approaches for OA treatment [3].

It is known that osteoblasts participate in the regulation of cartilage metabolism and bone remodeling in OA [4]. In particular, subchondral osteoblasts from OA patients show altered phenotypic characteristics [5, 6]. These cells are able to induce a phenotypic shift in OA chondrocytes towards the hypertrophic state [7] as well as the production of matrix metalloproteinases and the inhibition of aggrecan synthesis [8] which play an important role in cartilage degradation [9]. In addition, sites more distal to the joint articular surface show more rigid trabecular bone structure and lower mineralization related to an altered state of trabecular bone remodeling [10].

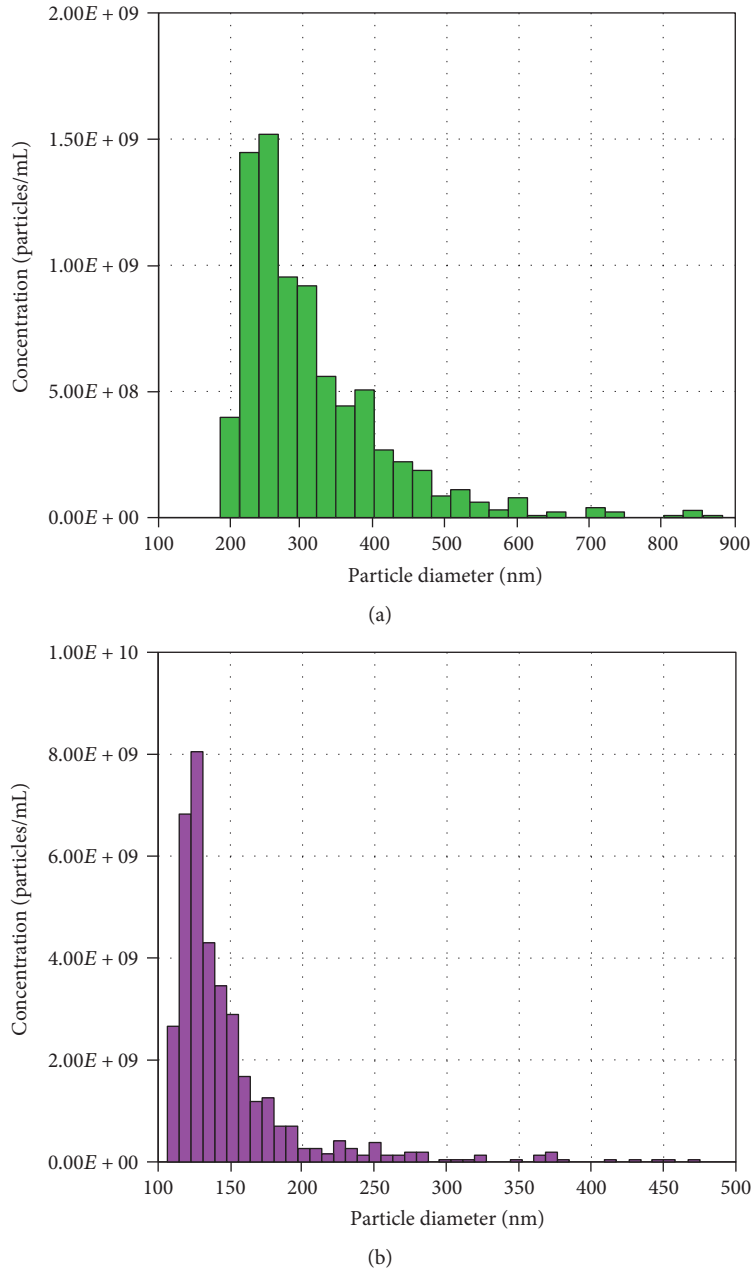


Figure 1: Characterization of MV and EX isolated from CM. Representative TRPS analysis of MV (a) and EX (b).

Proinflammatory cytokines are elevated in synovial fluid, synovial membrane, cartilage, and subchondral bone and have synergistic effects on inflammation, cartilage degradation, and bone remodeling in OA and diseases characterized by bone loss [11–13]. Interleukin- (IL-) 1β and tumor necrosis factor-α (TNFα) are thought to enhance osteoclastogenesis and bone resorption but they inhibit osteoblast differentiation and bone formation [13, 14]. Additionally, chronic inflammation can lead to cellular senescence in OA [15]. As a model of inflammatory stress, IL-1β stimulation of OA osteoblasts results in metabolic changes and the production of inflammatory and catabolic mediators as well as senescence features [16].

Novel therapeutic approaches for OA are being investigated as there is no pharmacological treatment able to modify the joint structural alterations. Some examples can be the injection of autologous and allogeneic mesenchymal stem cells or the differentiation into cartilage using scaffolds (reviewed in [17]). A wide range of evidence has shown the interest of adipose-derived mesenchymal stem cells (ASC) in tissue regeneration and cytoprotection. For instance, the administration of ASC into the knee joint inhibited synovial activation and prevented cartilage damage in experimental OA [18, 19]. The cytoprotective and anti-inflammatory properties of ASC in human chondrocytes and experimental OA may be mediated by paracrine effects [20–22] which are

also responsible for the inhibition of senescence in OA chondrocytes [23].

There is an increasing interest to know the properties of extracellular vesicles as novel ways of cellular communication [24]. The conditioned medium (CM) of ASC contains extracellular vesicles, mainly microvesicles (MV), and exosomes (EX), which may contribute to the paracrine effects of ASC. In this study, we have extended our previous observations in OA chondrocyte senescence [23] to investigate the contribution of extracellular vesicles to the paracrine effects of ASC on the cellular stress leading to senescence in OA osteoblasts.

2. Materials and Methods

2.1. Adipose-Derived Mesenchymal Stem Cells. ASC were isolated from the adipose tissue of 8 abdominoplasty-undergone healthy donors (2 men and 6 women, aged 54.4 ± 14.1 years, mean ± SEM). The experimental design was approved by the Institutional Ethical Committees (University of Valencia and La Fe Polytechnic University Hospital, Valencia, Spain). Samples were obtained from donors after they provided informed consent according to the Helsinki Declaration of 1975, as revised in 2013.

Samples were washed with phosphate-buffered saline (PBS) and minced and digested at 37°C for 1 h with 2% of type I collagenase (Gibco, Life Technologies, Madrid, Spain). Tissue remains were filtered through a 100 μm cell strainer (BD Biosciences Durham, NC, USA). Cells were then washed with DMEM/HAM F12 (Sigma-Aldrich, St. Louis, MO, USA) containing penicillin and streptomycin (1%), seeded onto tissue culture flasks ($1\text{-}2 \times 10^6$ cells/mL) in DMEM/HAM F12 medium with penicillin and streptomycin (1%), supplemented with 15% extracellular vesicle-free human serum, and incubated at 37°C in a 5% CO_2 atmosphere. Human serum was obtained from whole-blood donations of AB-blood-group-typed donors according to the criteria of Valencia Transfusion Centre. To eliminate the extracellular vesicle fraction, serum was centrifuged during 18 h at 120,000 ×g and 4°C using a SW-28 swinging-bucket rotor (Beckman Coulter, CA, USA). When cells reached semiconfluence, culture plates were washed and the ASC phenotype confirmed by flow cytometry (Flow Cytometer II, BD Biosciences, San Jose, CA, USA) using specific antibodies, anti-CD105-PE, antiCD90PerCP-eFluo 710, anti-CD34APC (eBioscience Inc., San Diego, CA, USA), and anti-CD45-PE (BD Pharmingen), and measuring cellular viability with propidium iodide. Finally, conditioned medium (CM) was collected from ASC culture cells at passage 0 every 48 h of culture. It was pooled, centrifuged, and stored in sterile conditions at −80°C prior to further use.

2.2. Isolation of Extracellular Vesicles. Vesicles were obtained from the CM of ASC using a filtration/centrifugation-based protocol. Cellular debris was eliminated by centrifugation at 300 ×g for 10 min. Vesicles were then collected from the supernatant through differential centrifugation steps. CM was filtered through 800 nm filter (Merck, Darmstadt, Germany) and centrifuged at 12,200 ×g for 20 min at 4°C to pellet microvesicles. Then, supernatants were filtered through a 200 nm filter (Merck, Darmstadt, Germany) and centrifuged at 100,000 ×g for 90 min at 4°C. Pellets were washed once with sterile PBS, resuspended in 15 μL of PBS, and stored at −80°C until further use.

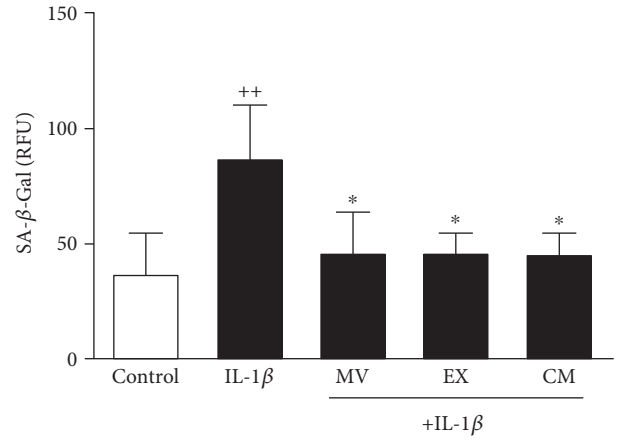
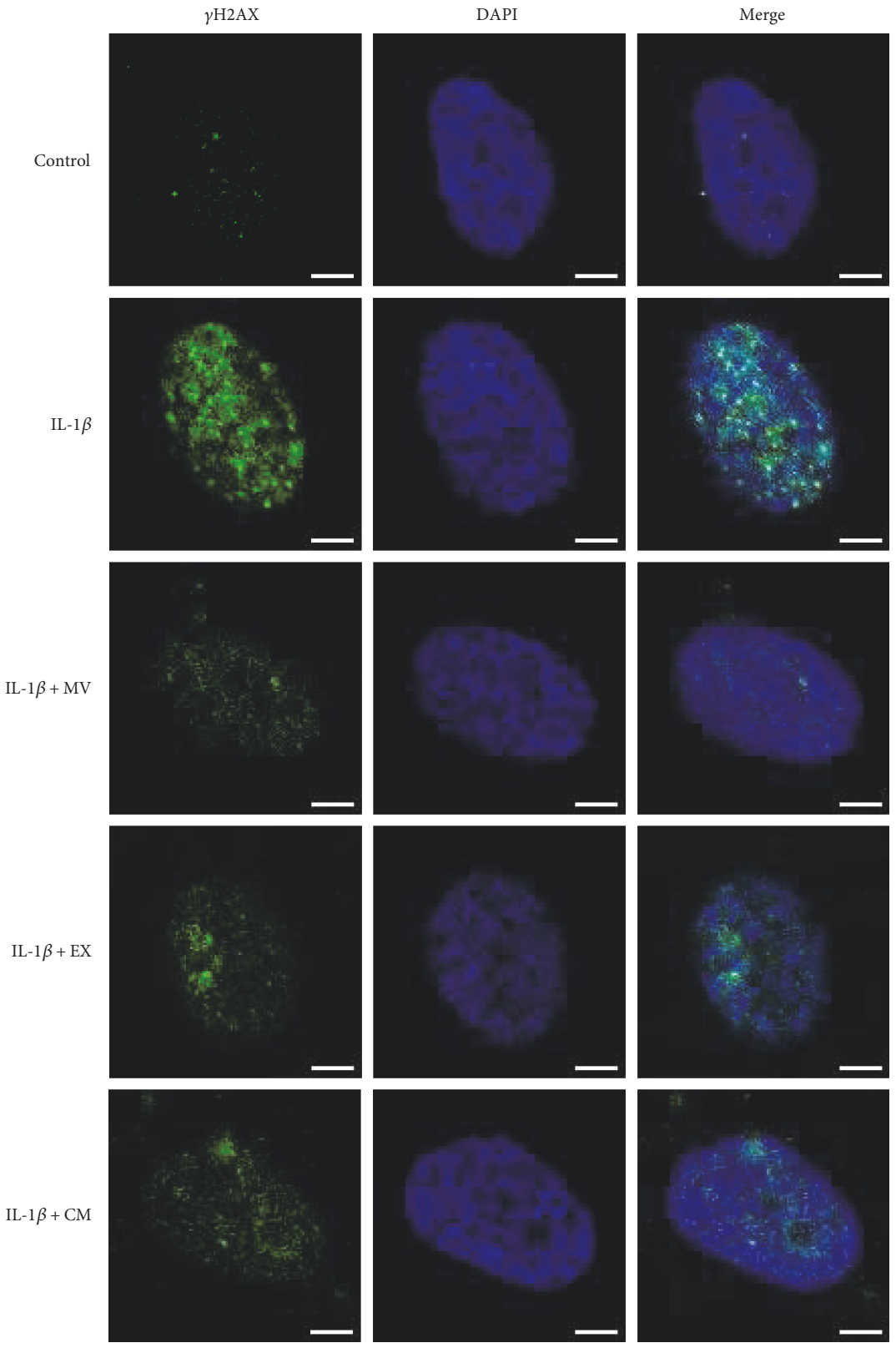


Figure 2: SA-β-Gal activity in OA osteoblasts. Cultures were treated with IL-1β alone or in combination with MV, EX, or CM for 7 days. SA-β-Gal activity was measured by using the cellular senescence assay kit (Cell Biolabs) and expressed as relative fluorescence units (RFU). Results show mean ± SD from 4 separate experiments with cells from separate donors. $^{++}P < 0.01$ compared to control (nonstimulated cells); $^{*}P < 0.05$ compared to IL-1β.

2.3. Tunable Resistive Pulse Sensing. Extracellular vesicle preparations were analyzed by Tunable Resistive Pulse Sensing (TRPS) using a qNano instrument (IZON Science Ltd., Oxford, UK) as previously described [25]. Briefly, NP100 and NP300 nanopore membranes were used to measure the samples of EX and MV, respectively. At least 500 events/sample were counted. Calibration was performed using calibration beads SKP200 and SKP400, provided by the manufacturer (IZON Science Ltd.).

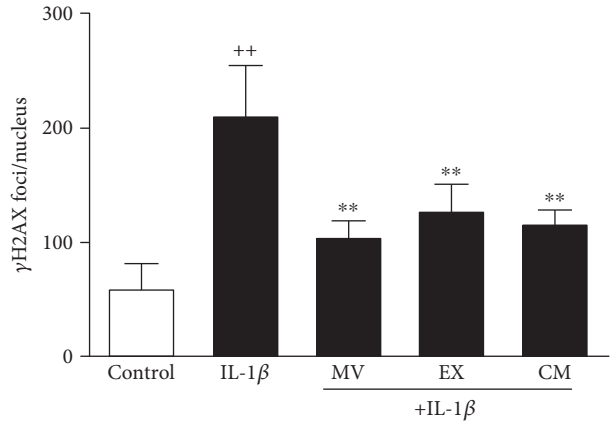
2.4. Transmission Electron Microscopy. Preparation of samples for transmission electron microscopy (TEM) was performed by the Microscopy Service (SCSIE, University of Valencia). LR-white resin inclusion was performed. Samples were filtered in resin and polymerized at 60°C for 48 h. Ultrathin slices (60 nm) were made with a diamond blade (DIATOME, Hartfield, USA) in eyelet grilles in a UC6 Ultracut (Leica, Wetzlar, Germany) and stained with uranyl acetate 2% for 25 min and lead citrate 3% for another 12 min prior to visualization in Jeol-1010 (JEOL Ltd. Tokyo, Japan) at 60 kV. Images were acquired with a digital camera MegaView III with Olympus Image Analysis Software (Olympus, Tokyo, Japan).

2.5. OA Osteoblasts. Knee specimens were obtained from patients with advanced OA diagnosed (21 women and 9 men, aged 68.4 ± 9.6 years, mean ± SEM) undergoing total knee joint replacement. Diagnosis was based on clinical and radiological evaluation. The experimental



(a)

Figure 3: Continued.



(b)

Figure 3: Immunofluorescence analysis of γH2AX foci accumulation. (a) Representative images. γH2AX foci (green, FITC fluorescence) and nuclei were stained with DAPI (blue). (b) Number of γH2AX foci per nucleus. Cultures were treated with IL-1β alone or in combination with MV, EX, or CM for 24 h. Bar = 5 μm. Results are expressed as mean ± SD from 3 separate experiments with cells from separate donors. $^{++}P < 0.01$ compared to control (nonstimulated cells); $^{**}P < 0.01$ compared to IL-1β.

design was approved by the institutional ethical committees (University of Valencia and La Fe Polytechnic University Hospital, Valencia, Spain). Samples were obtained from donors after they provided informed consent according to the Helsinki Declaration of 1975, as revised in 2013.

Trabecular bone samples were obtained from the femoral condyles and tibial plateaus, cut into small pieces, and subjected to enzymatic digestion with 1 mg/mL of collagenase type IA (Sigma-Aldrich) at 37°C in DMEM/HAM F-12 (Sigma-Aldrich), containing penicillin and streptomycin (1%) for 2 h. The digested tissue was cultured in osteoblast medium (Promocell, Labclinics S.A., Barcelona, Spain) in a humidified incubator with 5% CO_2 at 37°C. This medium was replaced twice a week. When cells were at 70% of confluence, bone fragments were removed and cells were allowed to grow until confluent. Cell phenotype was characterized by flow cytometry analysis using a Becton Dickinson FACSCanto II cytometer (BD, Franklin Lakes, NJ) and specific antibodies as previously reported [26]. For cell stimulation and treatment, subconfluent osteoblasts were incubated for 24 h in DMEM/HAM F12 (Sigma-Aldrich) containing penicillin and streptomycin (1%), supplemented with 15% extracellular vesicle-free human serum, and stimulated with IL-1β (10 ng/mL) in the presence or absence of MV (3.6×10^7 particles/mL), EX (7.2×10^7 particles/mL), or CM for 24 h (or 7 days for senescence-associated β-galactosidase activity (SA-β-Gal) experiments). These concentrations are in the range of those present in CM used in the same experiments.

2.6. Senescence-Associated β-Galactosidase Activity. Osteoblasts were seeded at 20×10^3 cells/well in Lab-Tek chambers (Thermo Scientific, Rochester, NY, USA), then stimulated with IL-1β (10 ng/mL), and treated with MV (3.6×10^7 particles/mL) or EX (7.2×10^7 particles/mL) or CM (0.2 mL) for 7 days. SA-β-Gal activity was measured using the cellular senescence assay kit from Cell Biolabs (San Diego, CA) in its fluorometric format. Briefly, cells were washed with cold PBS and lysed during 5 minutes at 4°C. Lysates were centrifuged and supernatant was collected as cell lysate. After transfer to fluorescence 96-well plates, lysates were incubated in the presence of assay buffer during 1 h at 37°C. Reaction was stopped and fluorescence was measured at 360 nm (excitation)/465 nm (emission) in a Victor3 microplate reader (PerkinElmer España, Madrid, Spain).

2.7. Immunofluorescence Assay for γH2AX Foci. Osteoblasts were seeded at 20×10^3 cells/well in Lab-Tek chambers (Thermo Scientific, Rochester, NY, USA), then stimulated with IL-1β (10 ng/mL), and treated with MV (3.6×10^7 particles/mL) or EX (7.2×10^7 particles/mL) or CM (0.2 mL) for 24 h. All cells were fixed with 4% formaldehyde in PBS for 30 min at 4°C and blocked with 5% normal goat serum and 0.3% Triton X-100 in PBS for 60 min at room temperature. Osteoblasts were further incubated with phospho-histone H2AX (Ser139) antibody (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. Finally, cells were incubated with FITC-conjugated goat antirabbit IgG (R&D Biosystems, Abingdon, UK), mounted in Prolong Gold antifade reagent with DAPI, and examined under a confocal microscope (Olympus FV1000, Tokyo, Japan).

2.8. Enzyme-Linked Immunosorbent Assay. Osteoblasts were stimulated with IL-1β (10 ng/mL) in the presence or absence of MV (3.6×10^7 particles/mL), EX (7.2×10^7 particles/mL), or CM (1 mL) for 24 h. Supernatants were harvested, centrifuged, and frozen at −80°C until analysis. In order to measure the levels of 4-hydroxy-nonenal (HNE) proteins, cells were lysed with 1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl, and 25 mM Tris, and pH 7.4 buffer. Lysates were centrifuged at 4°C for 10 min at 10,000 ×g. Then, 4-HNE-modified proteins were measured with the Cell Biolabs

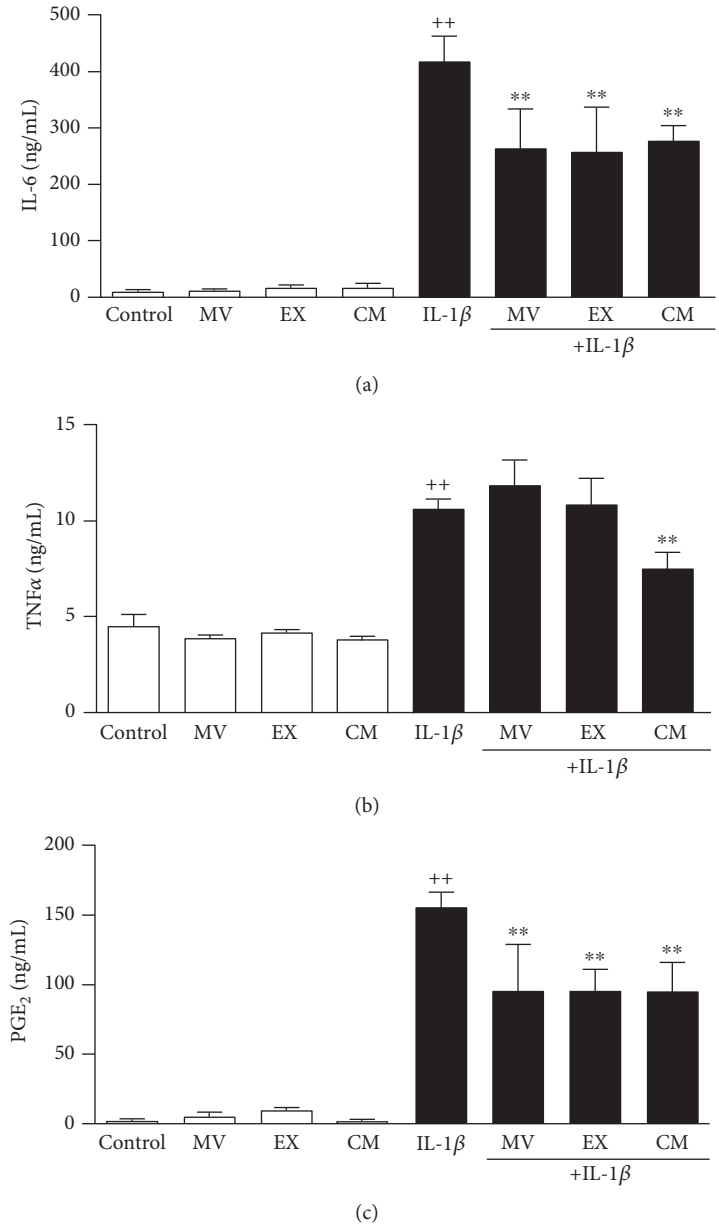


Figure 4: Release of inflammatory mediators. IL-6 (a) and TNFα (b) were measured by ELISA; PGE_2 (c) was measured by radioimmunoassay in cell culture supernatants of OA osteoblasts. Cultures were treated with IL-1β and/or MV, EX, or CM for 24 h. Results are expressed as mean ± SD from 4 separate experiments with cells from separate donors. $^{++}P < 0.01$ compared to control (nonstimulated cells); $^{**}P < 0.01$ compared to IL-1β.

ELISA kit (San Diego, CA, USA) with sensitivity of 1.56 μg/mL. TNFα, IL-6, and IL-10 were measured in supernatants with ELISA kits from eBioscience (San Diego, CA, USA) with a sensitivity of 4.0 pg/mL for TNFα and IL-6 and 2.0 pg/mL for IL-10.

2.9. Determination of Prostaglandin E_2. Osteoblasts were stimulated with IL-1β (10 ng/mL) in the presence or absence of MV (3.6×10^7 particles/mL), EX (7.2×10^7 particles/mL), or CM (1 mL) for 24 h. Supernatants were used to measure prostaglandin E_2 (PGE_2) by radioimmunoassay as previously described [27] using a Victor3 microplate reader (PerkinElmer España, Madrid, Spain).

2.10. Mitochondrial Membrane Potential. Osteoblasts were stimulated with IL-1β (10 ng/mL) in the presence or absence of MV (3.6×10^7 particles/mL), EX (7.2×10^7 particles/mL), or CM (1 mL) for 24 h. Then, mitochondrial transmembrane potential ($\Delta\psi$m) was assessed with the JC-1 probe (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolylcarbocyanine iodide, Thermo Scientific, Rochester, NY, USA). This lipophilic membrane-permeant cation

exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from ~525 nm (monomeric form) to ~590 nm (aggregated form). Cell cultures were trypsinized, resuspended in 1 mL of PBS, and incubated with 10 μg/mL of JC-1 dye for 15 min at 37°C and 5% CO_2. Both red and green fluorescence emissions were analyzed by flow cytometry using an excitation wavelength of 488 nm and observation wavelengths of 530 nm for green fluorescence and 585 nm for red fluorescence and a Becton Dickinson FACSCanto II cytometer (BD, Franklin Lakes, NJ, USA).

2.11. Statistical Analysis. The data were analyzed by one-way analysis of variance (ANOVA) followed by Sidak's posttest using the GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA). A P value of less than 0.05 was considered to be significant.

3. Results

3.1. Characterization of MV and EX from ASC. MV and EX fractions were isolated as indicated in Materials and Methods. TRPS analysis indicated a mean diameter of 316 nm and 115 nm and a concentration of 8×10^9 and 3.8×10^{10} particles/mL for MV and EX, respectively. Figure 1 shows a representative TRPS analysis of MV (a) and EX (b) fractions. The morphology of MV and EX was studied by TEM (data not shown).

3.2. SA-β-Gal Activity Induced by IL-1β in Human OA Osteoblasts. We examined SA-β-Gal activity in OA osteoblasts for the effects of CM and extracellular vesicles on this marker of cellular senescence. Figure 2 shows that IL-1β stimulation for 7 days enhanced SA-β-Gal activity by 57% with respect to control (nonstimulated cells). We found that treatment with MV, EX, or CM resulted in similar effects with a significant reduction of this activity by 48% with respect to IL-1β.

3.3. γH2AX Foci Accumulation. The presence of phosphorylated histone H2AX indicates DNA damage and correlates with age [28]. To assess the effect of CM and extracellular vesicles, γH2AX foci were quantified in nuclei. The immunofluorescence analysis showed that γH2AX foci were increased in the presence of IL-1β for 24 h by 70% compared with control (nonstimulated cells) (Figures 3(a) and 3(b)). The amount of γH2AX foci per nucleus was significantly reduced by treatment with MV (46%), EX (44%), or CM (30%).

3.4. Production of Proinflammatory and Anti-Inflammatory Mediators. Inflammation is involved in cellular senescence and OA. We have determined the production of key proinflammatory mediators in OA osteoblasts. After 24 h of incubation, IL-1β strongly induced the production of proinflammatory cytokine IL-6 and the eicosanoid PGE_2 while TNFα levels were enhanced to a lower extent (Figure 4). Treatment with MV, EX, or CM did not affect the basal release of these mediators. Nevertheless, MV, EX, and CM significantly reduced IL-6 and PGE_2, and CM also decreased

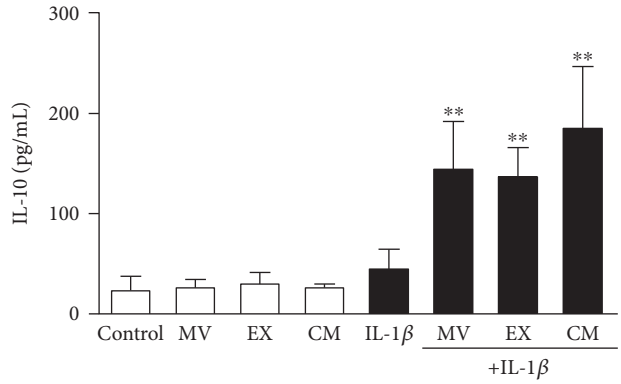


Figure 5: Release of IL-10 by OA osteoblasts. IL-10 was measured by ELISA in cell culture supernatants. Cultures were treated with IL-1β and/or MV, EX, or CM for 24 h. Results are expressed as mean ± SD from 5 separate experiments with cells from separate donors. $^{**}P < 0.01$ compared to IL-1β.

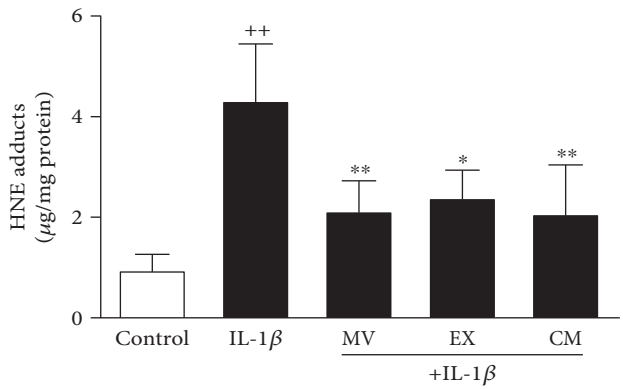


Figure 6: Quantification of HNE-protein adducts in OA osteoblasts. HNE-protein adducts were measured by ELISA in cellular extracts. Cultures were treated with IL-1β alone or in combination with MV, EX, or CM for 24 h. Results are expressed as mean ± SD from 4 separate experiments with cells from separate donors. $^{++}P < 0.01$ compared to control (nonstimulated cells); $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to IL-1β.

TNFα levels in cells stimulated with IL-1β. In addition, the anti-inflammatory cytokine IL-10 was measured in this system. As shown in Figure 5, after MV, EX, or CM treatment, the levels of IL-10 significantly increased by more than threefold after 24 h of incubation in the presence of IL-1β.

3.5. Oxidative Stress. As oxidative stress is a key process in the induction of cellular senescence [29], we next investigated the effects of CM, MV, and EX on protein modification by oxidative stress. As shown in Figure 6, IL-1β induced the production of oxidative stress leading to the accumulation of HNE-modified proteins in OA osteoblasts. We observed a significant reduction (by 50%) in the amount of HNE-protein adducts measured in cells treated with CM, MV, or EX.

3.6. Mitochondrial Membrane Potential. To measure changes in the mitochondrial membrane potential ($\Delta\Psi$), we have used the probe JC-1. Mitochondrial depolarization is

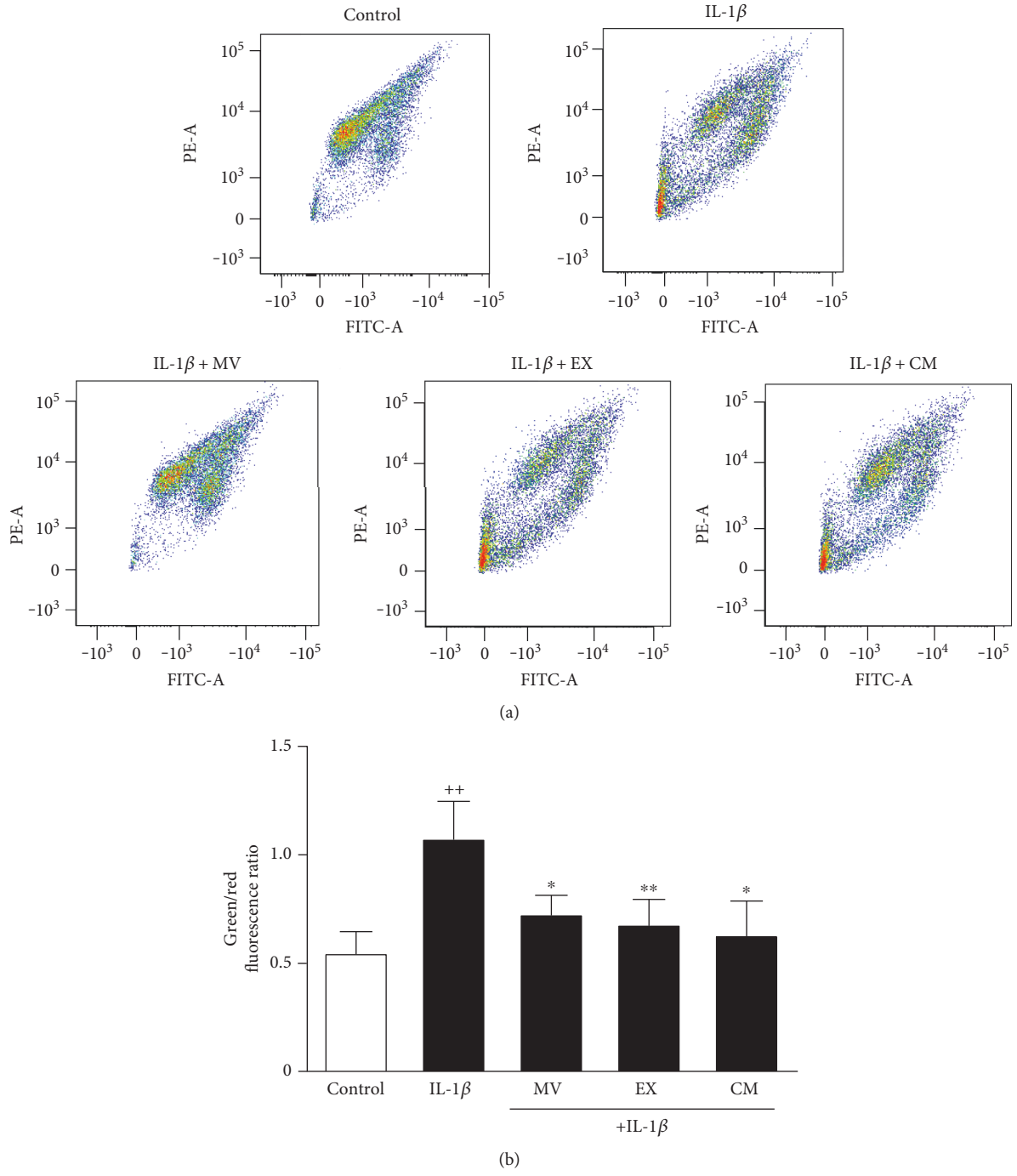


Figure 7: Analysis of mitochondrial membrane potential in OA osteoblasts. Analysis was performed by flow cytometry using the probe JC-1. Representative images (a); green/red fluorescence ratio (b). Cultures were treated with IL-1β alone or in combination with MV, EX, or CM for 24 h. Results are expressed as mean ± SD from 3 separate experiments with cells from separate donors. $^{++}P < 0.01$ compared to control (nonstimulated cells); $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to IL-1β.

indicated by a decrease in the red/green fluorescence intensity ratio. Incubation of OA osteoblasts with IL-1β increased the green/red ratio by twofold indicating a lowering of the mitochondrial membrane potential (Figure 7(a) and 7(b)). Treatment with CM, MV, or EX significantly restored the mitochondrial membrane potential.

4. Discussion

Multiple types of stress can lead to premature cellular senescence. It has been proposed that low-grade chronic inflammation during aging and associated pathologies can lead to oxidative stress and cell alteration-driving senescence.

Therefore, oxidative stress induces telomere-independent senescence leading to cell dysfunction [30]. Senescent cells develop a senescence-associated secretory phenotype with production of cytokines such as IL-6, growth factors, or matrix metalloproteinases which are mediators of complex autocrine and paracrine effects leading to phenotypic changes in nearby cells and alterations of tissue microenvironment [31]. Therefore, the accumulation of senescent cells with aging results in tissue or organ dysfunction. In support of this theory, it has been demonstrated that elimination of senescent cells in mice delays age-related pathologies [32].

There is increasing evidence that chronic inflammation-related senescence and aging may contribute to the development of OA [33]. The majority of studies of cellular senescence in OA have focused on chondrocytes. Chondrocyte senescence has been detected in OA cartilage [34] where the accumulation of cells could contribute to tissue destruction [35, 36]. ASC may offer new therapeutic approaches to regulate premature senescence. Recently, we have reported that ASC and CM inhibit senescence in OA chondrocytes [23]. In the current work, we have demonstrated the paracrine effects of ASC to downregulate senescence features induced by inflammatory stress in OA osteoblasts as well as the relevant contribution of MV and EX.

Joint tissues release proinflammatory cytokines in response to a wide variety of agents leading to mitochondrial changes, increased synthesis of reactive oxygen species (ROS), and DNA alterations which can induce premature senescence. In osteoblasts, cellular senescence is an important mechanism of age-related dysfunction which causes bone loss [37]. Aging bone shows a reduced ability of response against mechanical stress linked to some characteristics such as intralacunar hypermineralization and lower osteocyte lacunar density [38] which are also present in OA [39]. Subchondral bone alterations and cartilage degeneration are important processes during OA progression [40]. Interestingly, transplantation of senescent fibroblasts into the knee joint region of mice induces an inflammatory response and alterations in cartilage and bone resembling OA [41].

Proinflammatory and catabolic mediators produced by subchondral bone may contribute to cartilage and bone changes. It is considered that osteoblast cytokines can transmit the subchondral bone plate and calcified cartilage and communicate with chondrocytes [42]. Therefore, osteoblasts produce IL-6 which regulates the balance of bone resorption and formation during bone remodeling and can promote matrix degradation directly in both bone and cartilage [43]. We have demonstrated the paracrine anti-inflammatory effects of ASC on OA osteoblasts, with downregulation of IL-6 and TNFα. In addition, our results indicate that MV and EX could be the mediators of ASC paracrine effects on IL-6 which is the inflammatory marker showing the strongest association with age-related disease and fragility [33]. In contrast, MV and EX did not significantly reduce the levels of TNFα suggesting that soluble mediators present in CM may be the factors responsible for the regulation of this cytokine. The high levels of PGE_2 produced in our model of inflammatory stress were also reduced by CM and extracellular vesicles. The production of this eicosanoid is enhanced during cellular senescence in human fibroblasts [44]. Concerning bone metabolism, PGE_2 stimulates bone formation at low concentrations but it may be inhibitory at high concentrations [45, 46] and this eicosanoid may be a mediator of osteoclastogenesis induced by IL-6 [47]. In addition, PGE_2 may be an enhancing factor for IL-6 production in human osteoblasts [48]. Therefore, our results suggest that a decrease in PGE_2 production contributes to the anti-inflammatory and antisenescence effects of ASC and it may help to counteract the consequences of chronic inflammation on bone metabolism. In addition, we have shown that CM, MV, and EX from ASC enhance the production of the anti-inflammatory cytokine IL-10 in the presence of IL-1β which may prolong the downregulation of the inflammatory response as this cytokine inhibits the production of ROS and proinflammatory cytokines by macrophages [49, 50] and PGE_2 by OA synovial fibroblasts [51]. This effect of CM, MV, and EX on IL-10 is in line with that reported for CM in OA chondrocytes [52]. Of note, IL-10 has been proposed as a treatment option for inflammation-related bone loss [53].

Chronic oxidative stress related to aging or mechanical stress may lead to cellular senescence in joint tissues [54] and age-related alterations in osteoblast differentiation and function [37, 55]. The majority of ROS are produced by the mitochondria as a consequence of oxidative phosphorylation which generates a potential energy for protons ($\Delta\Psi$) across the mitochondrial inner membrane. ROS generated within the mitochondria can damage mitochondrial components and nuclear DNA, besides inducing the oxidative modification of proteins and the activation of different signaling pathways [56]. We have examined whether the control of oxidative stress could be involved in the protective effects of CM and extracellular vesicles observed in OA osteoblasts. The results of our analysis indicate that CM, MV, and EX from ASC significantly downregulate the mitochondrial membrane changes and oxidative stress induced by IL-1β, thus providing a plausible mechanism to inhibit cellular senescence. Mitochondrial ROS are linked to senescence through nuclear DNA damage [57]. The phosphorylation of H2AX following DNA double-strand breaks increases with age and may be a biomarker for human morbidity in age-related diseases [28]. We found that CM and extracellular vesicles from ASC are able to reduce DNA damage as shown by a lower accumulation of γH2AX foci which may be a consequence of oxidative stress control.

As osteoblasts play an important role in the regulation of cartilage metabolism and bone remodeling, the correction of the abnormal cell metabolism may offer novel therapeutic approaches for joint degradation. Further research into the mechanisms by which senescence of different articular cells contributes to OA is needed to uncover novel targets useful to prevent or treat this condition.

In conclusion, we have shown that CM and extracellular vesicles from ASC downregulate inflammation and oxidative stress which may mediate antisenescence effects in OA osteoblasts. Our data also indicate that MV and EX from ASC are responsible for the paracrine effects of these cells and suggest the interest of these extracellular vesicles to develop new treatments for joint conditions.

Conflicts of Interest

The authors have no conflicts of interest to disclose.

Authors' Contributions

Maria Isabel Guillén and Maria José Alcaraz contributed equally to this work.

Acknowledgments

This work has been funded by Grants SAF2013-48724-R (MINECO/FEDER) and PROMETEOII/2014/071 (Generalitat Valenciana).

References

[1] J. P. Mansell and A. J. Bailey, "Abnormal cancellous bone collagen metabolism in osteoarthritis," *The Journal of Clinical Investigation*, vol. 101, no. 8, pp. 1596–1603, 1998.

[2] Y. Henrotin, L. Pesesse, and C. Sanchez, "Subchondral bone and osteoarthritis: biological and cellular aspects," *Osteoporosis International*, vol. 23, Supplement 8, pp. S847–S851, 2012.

[3] C. Boileau, S. K. Tat, J. P. Pelletier, S. Cheng, and J. Martel-Pelletier, "Diacerein inhibits the synthesis of resorptive enzymes and reduces osteoclastic differentiation/survival in osteoarthritic subchondral bone: a possible mechanism for a protective effect against subchondral bone remodelling," *Arthritis Research & Therapy*, vol. 10, no. 3, p. R71, 2008.

[4] C. Westacott, "Interactions between subchondral bone and cartilage in OA. Cells from osteoarthritic bone can alter cartilage metabolism," *Journal of Musculoskeletal Neuronal Interactions*, vol. 2, no. 6, pp. 507–509, 2002.

[5] G. Hilal, J. Martel-Pelletier, J. P. Pelletier, P. Ranger, and D. Lajeunesse, "Osteoblast-like cells from human subchondral osteoarthritic bone demonstrate an altered phenotype in vitro: possible role in subchondral bone sclerosis," *Arthritis & Rheumatology*, vol. 41, no. 5, pp. 891–899, 1998.

[6] D. Couchourel, I. Aubry, A. Delalandre et al., "Altered mineralization of human osteoarthritic osteoblasts is attributable to abnormal type I collagen production," *Arthritis & Rheumatism*, vol. 60, no. 5, pp. 1438–1450, 2009.

[7] C. Sanchez, M. A. Deberg, N. Piccardi, P. Msika, J. Y. Reginster, and Y. E. Henrotin, "Subchondral bone osteoblasts induce phenotypic changes in human osteoarthritic chondrocytes," *Osteoarthritis and Cartilage*, vol. 13, no. 11, pp. 988–997, 2005.

[8] C. Sanchez, M. A. Deberg, N. Piccardi, P. Msika, J. Y. Reginster, and Y. E. Henrotin, "Osteoblasts from the sclerotic subchondral bone downregulate aggrecan but upregulate metalloproteinases expression by chondrocytes. This effect is mimicked by interleukin-6, -1β and oncostatin M pre-treated non-sclerotic osteoblasts," *Osteoarthritis and Cartilage*, vol. 13, no. 11, pp. 979–987, 2005.

[9] C. I. Westacott, G. R. Webb, M. G. Warnock, J. V. Sims, and C. J. Elson, "Alteration of cartilage metabolism by cells from osteoarthritic bone," *Arthritis & Rheumatism*, vol. 40, no. 7, pp. 1282–1291, 1997.

[10] D. D. Kumarasinghe, E. Perilli, H. Tsangari et al., "Critical molecular regulators, histomorphometric indices and their correlations in the trabecular bone in primary hip osteoarthritis," *Osteoarthritis and Cartilage*, vol. 18, no. 10, pp. 1337–1344, 2010.

[11] H. Hulejova, V. Baresova, Z. Klezl, M. Polanska, M. Adam, and L. Senolt, "Increased level of cytokines and matrix metalloproteinases in osteoarthritic subchondral bone," *Cytokine*, vol. 38, no. 3, pp. 151–156, 2007.

[12] P. Wojdasiewicz, L. A. Poniatowski, and D. Szukiewicz, "The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis," *Mediators of Inflammation*, vol. 2014, Article ID 561459, 19 pages, 2014.

[13] P. Lencel and D. Magne, "Inflammaging: the driving force in osteoporosis?," *Medical Hypotheses*, vol. 76, no. 3, pp. 317–321, 2011.

[14] D. C. Lacey, P. J. Simmons, S. E. Graves, and J. A. Hamilton, "Proinflammatory cytokines inhibit osteogenic differentiation from stem cells: implications for bone repair during inflammation," *Osteoarthritis and Cartilage*, vol. 17, no. 6, pp. 735–742, 2009.

[15] R. F. Loeser, "Aging processes and the development of osteoarthritis," *Current Opinion in Rheumatology*, vol. 25, no. 1, pp. 108–113, 2013.

[16] V. Clerigues, M. I. Guillen, M. A. Castejon, F. Gomar, V. Mirabet, and M. J. Alcaraz, "Heme oxygenase-1 mediates protective effects on inflammatory, catabolic and senescence responses induced by interleukin-1β in osteoarthritic osteoblasts," *Biochemical Pharmacology*, vol. 83, no. 3, pp. 395–405, 2012.

[17] Y. M. Pers, M. Ruiz, D. Noël, and C. Jorgensen, "Mesenchymal stem cells for the management of inflammation in osteoarthritis: state of the art and perspectives," *Osteoarthritis and Cartilage*, vol. 23, no. 11, pp. 2027–2035, 2015.

[18] M. ter Huurne, R. Schelbergen, R. Blattes et al., "Antiinflammatory and chondroprotective effects of intraarticular injection of adipose-derived stem cells in experimental osteoarthritis," *Arthritis and Rheumatism*, vol. 64, no. 11, pp. 3604–3613, 2012.

[19] G. Desando, C. Cavallo, F. Sartoni et al., "Intra-articular delivery of adipose derived stromal cells attenuates osteoarthritis progression in an experimental rabbit model," *Arthritis Research & Therapy*, vol. 15, no. 1, p. R22, 2013.

[20] C. Manferdini, M. Maumus, E. Gabusi et al., "Adipose-derived mesenchymal stem cells exert antiinflammatory effects on chondrocytes and synoviocytes from osteoarthritis patients through prostaglandin E_2," *Arthritis & Rheumatism*, vol. 65, no. 5, pp. 1271–1281, 2013.

[21] M. Maumus, C. Manferdini, K. Toupet et al., "Adipose mesenchymal stem cells protect chondrocytes from degeneration associated with osteoarthritis," *Stem Cell Research*, vol. 11, no. 2, pp. 834–844, 2013.

[22] K. Kuroda, T. Kabata, K. Hayashi et al., "The paracrine effect of adipose-derived stem cells inhibits osteoarthritis progression," *BMC Musculoskeletal Disorders*, vol. 16, no. 1, p. 236, 2015.

[23] J. Platas, M. I. Guillen, M. D. Perez Del Caz et al., "Paracrine effects of human adipose-derived mesenchymal stem cells in inflammatory stress-induced senescence features of osteoarthritic chondrocytes," *Aging*, vol. 8, no. 8, pp. 1703–1717, 2016.

[24] M. Colombo, G. Raposo, and C. Théry, "Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles," *Annual Review of Cell and Developmental Biology*, vol. 30, pp. 255–289, 2014.

[25] R. Vogel, F. A. Coumans, R. G. Maltesen et al., "A standardized method to determine the concentration of extracellular

vesicles using tunable resistive pulse sensing," *Journal of Extracellular Vesicles*, vol. 5, no. 1, article 31242, 2016.

[26] J. Platas, M. I. Guillen, F. Gomar, M. A. Castejon, P. Esbrit, and M. J. Alcaraz, "Anti-senescence and anti-inflammatory effects of the C-terminal moiety of PTHrP peptides in OA osteoblasts," *Journal of Gerontology Series A*, vol. 72, no. 5, pp. 624–631, 2017.

[27] M. A. Moroney, M. J. Alcaraz, R. A. Forder, F. Carey, and J. R. S. Hoult, "Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids," *Journal of Pharmacy and Pharmacology*, vol. 40, no. 11, pp. 787–792, 1988.

[28] S. H. Schurman, C. A. Dunn, R. Greaves et al., "Age-related disease association of endogenous γ-H2AX foci in mononuclear cells derived from leukapheresis," *PLoS One*, vol. 7, no. 9, article e45728, 2012.

[29] I. Ben Porath and R. A. Weinberg, "The signals and pathways activating cellular senescence," *International Journal of Biochemistry & Cell Biology*, vol. 37, no. 5, pp. 961–976, 2005.

[30] K. Itahana, J. Campisi, and G. P. Dimri, "Mechanisms of cellular senescence in human and mouse cells," *Biogerontology*, vol. 5, no. 1, pp. 1–10, 2004.

[31] T. Tchkonia, Y. Zhu, D. J. Van, J. Campisi, and J. L. Kirkland, "Cellular senescence and the senescent secretory phenotype: therapeutic opportunities," *The Journal of Clinical Investigation*, vol. 123, no. 3, pp. 966–972, 2013.

[32] D. J. Baker, T. Wijshake, T. Tchkonia et al., "Clearance of $p16^{Ink4a}$-positive senescent cells delays ageing-associated disorders," *Nature*, vol. 479, no. 7372, pp. 232–236, 2011.

[33] M. A. Greene and R. F. Loeser, "Aging-related inflammation in osteoarthritis," *Osteoarthritis and Cartilage*, vol. 23, no. 11, pp. 1966–1971, 2015.

[34] J. S. Price, J. G. Waters, C. Darrah et al., "The role of chondrocyte senescence in osteoarthritis," *Aging Cell*, vol. 1, no. 1, pp. 57–65, 2002.

[35] R. F. Loeser, "Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix," *Osteoarthritis and Cartilage*, vol. 17, no. 8, pp. 971–979, 2009.

[36] D. Philipot, D. Guerit, D. Platano et al., "$p16^{INK4a}$ and its regulator miR-24 link senescence and chondrocyte terminal differentiation-associated matrix remodelling in osteoarthritis," *Arthritis Research & Therapy*, vol. 16, no. 1, p. R58, 2014.

[37] M. Kassem and P. J. Marie, "Senescence-associated intrinsic mechanisms of osteoblast dysfunctions," *Aging Cell*, vol. 10, no. 2, pp. 191–197, 2011.

[38] B. Busse, D. Djonic, P. Milovanovic et al., "Decrease in the osteocyte lacunar density accompanied by hypermineralized lacunar occlusion reveals failure and delay of remodeling in aged human bone," *Aging Cell*, vol. 9, no. 6, pp. 1065–1075, 2010.

[39] V. T. Carpentier, J. Wong, Y. Yeap et al., "Increased proportion of hypermineralized osteocyte lacunae in osteoporotic and osteoarthritic human trabecular bone: implications for bone remodeling," *Bone*, vol. 50, no. 3, pp. 688–694, 2012.

[40] C. I. Westacott, A. F. Barakat, L. Wood et al., "Tumor necrosis factor alpha can contribute to focal loss of cartilage in osteoarthritis," *Osteoarthritis and Cartilage*, vol. 8, no. 3, pp. 213–221, 2000.

[41] M. Xu, E. W. Bradley, M. M. Weivoda et al., "Transplanted senescent cells induce an osteoarthritis-like condition in mice," *The Journals of Gerontology Series A*, vol. 72, no. 6, pp. 780–785, 2017.

[42] D. M. Findlay and G. J. Atkins, "Osteoblast-chondrocyte interactions in osteoarthritis," *Current Osteoporosis Reports*, vol. 12, no. 1, pp. 127–134, 2014.

[43] K. Sakao, K. A. Takahashi, O. Mazda et al., "Enhanced expression of interleukin-6, matrix metalloproteinase-13, and receptor activator of NF-κB ligand in cells derived from osteoarthritic subchondral bone," *Journal of Orthopedic Science*, vol. 13, no. 3, pp. 202–210, 2008.

[44] S. Martien, O. Pluquet, C. Vercamer et al., "Cellular senescence involves an intracrine prostaglandin E_2 pathway in human fibroblasts," *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, vol. 1831, no. 7, pp. 1217–1227, 2013.

[45] L. G. Raisz, "Prostaglandins and bone: physiology and pathophysiology," *Osteoarthritis and Cartilage*, vol. 7, no. 4, pp. 419–421, 1999.

[46] K. A. Blackwell, L. G. Raisz, and C. C. Pilbeam, "Prostaglandins in bone: bad cop, good cop?," *Trends in Endocrinology & Metabolism*, vol. 21, no. 5, pp. 294–301, 2010.

[47] H. Tai, C. Miyaura, C. C. Pilbeam et al., "Transcriptional induction of cyclooxygenase-2 in osteoblasts is involved in interleukin-6-induced osteoclast formation," *Endocrinology*, vol. 138, no. 6, pp. 2372–2379, 1997.

[48] F. Massicotte, D. Lajeunesse, M. Benderdour et al., "Can altered production of interleukin-1β, interleukin-6, transforming growth factor-β and prostaglandin E_2 by isolated human subchondral osteoblasts identify two subgroups of osteoarthritic patients," *Osteoarthritis and Cartilage*, vol. 10, no. 6, pp. 491–500, 2002.

[49] S. Dokka, X. Shi, S. Leonard, L. Wang, V. Castranova, and Y. Rojanasakul, "Interleukin-10-mediated inhibition of free radical generation in macrophages," *American Journal of Physiology - Lung Cellular and Molecular Physiology*, vol. 280, no. 6, pp. L1196–L1202, 2001.

[50] D. F. Fiorentino, A. Zlotnik, T. R. Mosmann, M. Howard, and A. O'Garra, "IL-10 inhibits cytokine production by activated macrophages," *Journal of Immunology*, vol. 147, no. 11, pp. 3815–3822, 1991.

[51] N. Alaaeddine, J. A. Di Battista, J. P. Pelletier, K. Kiansa, J. M. Cloutier, and J. Martel-Pelletier, "Inhibition of tumor necrosis factor α-induced prostaglandin E_2 production by the antiinflammatory cytokines interleukin-4, interleukin-10, and interleukin-13 in osteoarthritic synovial fibroblasts: distinct targeting in the signaling pathways," *Arthritis & Rheumatology*, vol. 42, no. 4, pp. 710–718, 1999.

[52] J. Platas, M. I. Guillen, M. D. del Caz, F. Gomar, V. Mirabet, and M. J. Alcaraz, "Conditioned media from adipose tissue-derived mesenchymal stem cells down-regulate degradative mediators induced by interleukin-1β in osteoarthritic chondrocytes," *Mediators of Inflammation*, vol. 2013, Article ID 357014, 10 pages, 2013.

[53] Q. Zhang, B. Chen, F. Yan et al., "Interleukin-10 inhibits bone resorption: a potential therapeutic strategy in periodontitis and other bone loss diseases," *BioMed Research International*, vol. 2014, Article ID 284836, 5 pages, 2014.

[54] K. McCulloch, G. J. Litherland, and T. S. Rai, "Cellular senescence in osteoarthritis pathology," *Aging Cell*, vol. 16, no. 2, pp. 210–218, 2017.

[55] M. Almeida, L. Han, M. Martin-Millan et al., "Skeletal involution by age-associated oxidative stress and its acceleration by loss of sex steroids," *Journal of Biological Chemistry*, vol. 282, no. 37, pp. 27285–27297, 2007.

[56] R. S. Balaban, S. Nemoto, and T. Finkel, "Mitochondria, oxidants, and aging," *Cell*, vol. 120, no. 4, pp. 483–495, 2005.

[57] J. F. Passos, G. Nelson, C. Wang et al., "Feedback between p21 and reactive oxygen production is necessary for cell senescence," *Molecular Systems Biology*, vol. 6, p. 347, 2010.

Biochemical Pharmacology 153 (2018) 134–146

Contents lists available at ScienceDirect

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Review

Extracellular vesicles: A new therapeutic strategy for joint conditions

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ARTICLE INFO

Keywords:
Extracellular vesicles
Mesenchymal stem cells
Inflammation
Immunomodulation
Rheumatoid arthritis
Osteoarthritis

ABSTRACT

Extracellular vesicles (EVs) are attracting increasing interest since they might represent a more convenient therapeutic tool with respect to their cells of origin. In the last years much time and effort have been expended to determine the biological properties of EVs from mesenchymal stem cells (MSCs) and other sources. The immunoregulatory, anti-inflammatory and regenerative properties of MSC EVs have been demonstrated in *in vitro* studies and animal models of rheumatoid arthritis or osteoarthritis. This cell-free approach has been proposed as a possible better alternative to MSC therapy in autoimmune conditions and tissue regeneration. In addition, EVs show great potential as biomarkers of disease or delivery systems for active molecules. The standardization of isolation and characterization methods is a key step for the development of EV research. A better understanding of EV mechanisms of action and efficacy is required to establish the potential therapeutic applications of this new approach in joint conditions.

1. Introduction

Extracellular vesicles (EVs) are actively secreted by cells and represent a mechanism for cell-to-cell signaling in physiological and pathophysiological responses [1,2]. These microparticles are usually classified based on the mode of biogenesis as microvesicles, exosomes, and apoptotic bodies [3]. Microvesicles and exosomes are both commonly found in extracellular fluids and represent the most described classes of EVs. Microvesicles are shedding vesicles between 50 nm and 1 μm in diameter generated by plasma membrane protrusions followed by fission of their membrane stalk [3,4]. Exosomes are formed as intraluminal vesicles in endosomal compartments called multivesicular bodies and they are released in an exocytic manner by fusion of these multivesicular endosomes with the plasma membrane. These EVs show a mean size of 40–100 nm in diameter and are enriched in endosome related proteins [4,5] whereas apoptotic bodies (50–5000 nm in diameter) are released from fragmented apoptotic cells [3].

Joint conditions represent an important public health problem as they are a major cause of pain, functional limitation and physical disability. As a main example, rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial angiogenesis, hyperplasia of the synovial membrane and infiltration of immune cells besides cartilage damage and bone resorption [6]. RA is a systemic disease which can affect organs such as the lungs, heart and eyes and is associated with an increased risk of cardiovascular disease, infection, lymphoma, and reduced life expectancy [7,8]. The cause of RA is not yet fully understood, although autoimmune dysfunction plays a leading role in inflammation and joint damage, with a pre-rheumatoid phase preceding the onset of articular disease followed by established RA. Modification of the abnormal immune response by immune modulatory cells and other novel approaches represents an attractive possibility to achieve long-term tolerance and control of chronic inflammation [9].

Osteoarthritis (OA), the most prevalent joint condition in the elderly, is associated with progressive articular cartilage loss, low-grade synovitis and alterations in subchondral bone and periarticular tissues. There is an imbalance between anabolic and catabolic processes in the joint as well as a relevant contribution of mechanical stress and inflammatory mediators [10]. A number of risk factors are associated with OA, such as advancing age, obesity, and trauma that determine the progression of pathophysiological events in joint tissues [11]. There is no effective treatment for OA although new therapies to stop disease progression or repair tissue damage are been investigated.

In the last years, the interest for the therapeutic applications of EVs has exponentially increased as these microparticles may reproduce the effects of parent cells with some advantages. In particular, EVs from mesenchymal stem cells (MSCs) provide a promising approach for immunomodulation and tissue regeneration. How these effects are mediated are not yet clear but EVs represent a novel strategy for future cell-free therapy of joint pathologies. Although these studies are at an early stage, the possible activity of EVs in joint conditions is of great interest

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https://doi.org/10.1016/j.bcp.2018.02.004
Received 27 November 2017; Accepted 5 February 2018
Available online 07 February 2018
0006-2952/

Table 1
Best established methods for the isolation of EVs [12–18].

Method	Principle	Scalability	Advantages	Challenges
Precipitation	Precipitant agent	Yes	Fast Simple	Low purity Protein contaminations Precipitant interferences
Differential ultracentrifugation	Size	No	Common EV subfractioning	EV aggregation Possible loss of function
Density gradient ultracentrifugation	Density	No	Common EV subfractioning High purity	Gradients may interfere with EVs' activity Possible loss of function Time consuming
Ultrafiltration	Size	Yes	High concentration	Aggressive Bias towards pressure-resisting EVs
Size exclusion chromatography	Size	Yes	High purity Removal of soluble proteins	Low yield Need of further concentration steps
Immunoaffinity	EV phenotype	No	Fast High purity	Low yield Expensive Bias towards known markers-containing EVs

and will be the focus of this review.

2. Isolation and characterization of extracellular vesicles

Current methods for isolating exosomes from biologic fluids include differential ultracentrifugation, density gradient centrifugation, size exclusion chromatography, polymer-based precipitation, filtration and immunoaffinity capture, as summarized in Table 1. All of them have limitations such as co-isolation of contaminating materials, loss of EV components due to damaged membrane integrity during isolation or failure to completely isolate EV fractions. In particular, removal of serum proteins and lipoproteins is problematic [12]. Depletion of the most abundant serum proteins such as albumin or immunoglobulins seems necessary to avoid biasing downstream analysis. Sample collection from different biological fluids should take into consideration possible sources of artefacts and variability [13]. Platelet removal, for example, is mandatory when working with blood, plasma or serum, as platelets release EVs upon activation in freeze-thaw cycles [14]. Additionally, serum used to supplement culture media must be previously EV depleted [15]. Therefore the presence of contaminants may influence the behavior of EV preparations leading to confusing effects on target cells. In addition, EVs from different sources can exhibit differences in composition or in non-specific component aggregation to their surface which can alter their physicochemical properties [16] and diverse EV subpopulations can be secreted by the same cell [17]. Consistency of pre-analytical procedures and report of complete experimental details have been recommended in order to get reproducible results [12].

There has been a great improvement of detection technologies during the past 20 years [18]. Quantification of EVs is usually performed by nanoparticle tracking analysis, tuneable resistive pulse sensing [19] or dynamic light scattering, and morphology confirmed by transmission electron microscopy, cryo-electron microscopy or atomic force microscopy [12]. Determinations of protein to lipid ratio, lipid bilayer order, and lipid composition may prove useful for quality control of EVs [20]. Western blotting or flow cytometry with fluorescent counting beads are normally used to detect EV protein markers [21]. The detection of specific markers would include CD63, CD9, and CD81 tetraspanins and endosome markers such as syntenin-1, ALG-2-interacting protein X (Alix) and tumor susceptibility gene 101 protein (TSG101), for exosomes [4,5,22]. Microvesicles can include cytoskeletal components (actin, actin-binding proteins (profilin-1, cofilin-1), myosin, tubulin), enzymes (alpha-enolase, pyruvate kinase, triosephosphate isomerase), membrane molecules (HLA-I, HLA-II antigens, Na^+/K^+ ATPase), proteins involved in vesicle biogenesis and trafficking (e.g. Ras-related proteins), lactadherin that binds to the phosphatidyl-serine surface of microvesicles, or clusterin (ApoJ), a protein involved in the clearance of apoptotic bodies and cell debris [21]. In addition, a set of components is cell-specific. Therefore, EVs from MSCs express on their surface MSC markers CD29, CD73, CD44 and CD105, as well as cell adhesion molecules and growth factor receptors. Inside EVs, a wide range of active molecules can be found such as cytokines, enzymes, nuclear receptors, miRNAs and other RNAs such as transcription factor CP2/clock homolog, retinoblastoma-like-1, ubiquitin-related modifier-1 and interleukin-1 receptor antagonist [23].

Studies in 3T3-L1 mature adipocytes have shown a role for protein and lipid content in the characterization of large EVs (probably including microvesicles, with expression of β-actin and enrichment in endoplasmin and α-actinin-4), and small EV populations (sEVs, with expression of exosomal markers Alix, TSG101 and tetraspanins). The lipidomic analysis indicated cholesterol enrichment of sEVs, whereas large EVs were characterized by high amounts of externalized phosphatidylserine [24]. It has also been demonstrated the presence of two distinct subpopulations of exosomes (low density fractions exosomes and high density fractions exosomes). Both types express the exosomal markers Alix and TSG101 but differed in the presence of α-actinin-4, cyclin-Y (enriched in low density fraction exosomes) and ephrin type-A receptor 2 proteins (enriched in high density fractions exosomes) as well as in their RNA content [17]. As isolation methods based on different biogenesis pathways are still lacking, an universal nomenclature has been proposed based exclusively on size: large EVs pelleted at low speed, medium-sized EVs pelleted at intermediate speed, and sEVs pelleted at high speed. Among sEVs, further subcategories may be distinguished based on the presence or absence of different markers: a, enriched in CD63, CD9 and CD81 tetraspanins and endosome markers; b, devoid of CD63 and CD81 but enriched in CD9; and two groups not associated to the endosomal pathway: c, devoid of CD63/CD9/CD81; and d, enriched in extracellular matrix or serum-derived factors [22].

There is an increasing interest in the structural and functional biology of EVs. In addition to common components [25], these microparticles contain markers from the parent cells and therefore cell type specific protein, mRNA, miRNA, and lipid subsets have been identified which can be useful for diagnostic and therapeutic purposes. Interestingly, stress conditions or activation of intracellular signaling by mediators such as cytokines change EV composition and therefore the response of recipient cells [26–28]. The content of proteins, RNA and lipids has been investigated by high-throughput methods. Genomic DNA has also been detected in EVs although its function is unknown [28]. In addition to classical techniques, proteomic analyses of EVs can be performed by high-resolution and high-sensitivity mass spectrometry and high-resolution liquid chromatography mass-spectrometry-based approaches [29]. These techniques and gas chromatography coupled to mass spectrometry, provide information on the presence of lipid species and metabolites in EVs [30]. EVs contain lipids in a bilayer membrane

and also transport bioactive lipids and lipid related enzymes such as phospholipase A_2 and other enzymes involved in eicosanoid synthesis. Besides, EVs are enriched in cholesterol and sphingomyelin which can accumulate in recipient cells [31] as EVs may transfer lipids between cells for metabolism into bioactive mediators [32]. Metabolomic strategies have recently provided the characterization of EV metabolic activity [30]. Interestingly, high throughput transcriptomic studies have identified a wide range of mRNA and miRNA data sets based on microarray and next-generation sequencing analyses leading to a comprehensive data classification [33,34]. These EV components can be functional after transfer to cells [35]. Other RNA species within EVs include viral RNSs, Y-RNAs, fragments of tRNAs, small nuclear RNA, small nucleolar RNA, piwi-interacting RNAs, long non-coding RNAs and circular RNAs [28,36]. There are a wide range of studies on EV composition which are collected by three curated data repositories: ExoCarta [37], Vesiclepedia [3] and EVpedia [38], and functional enrichment analysis tools are also available [39].

3. Immunomodulatory effects of extracellular vesicles

A wide range of evidence indicates that EVs produced by both immune and non-immune cells can play an important role in the regulation of immunity (reviewed in [40,41]). Circulating endogenous EVs produced by different cell types contribute to the suppression of immune responses, either in an antigen-specific or a nonspecific manner. For instance, platelet-derived EVs can inhibit inflammatory responses due to the presence of 12-lipoxygenase which is transferred to mast cells to synthesize the pro-resolving mediator lipoxin A_4 [42]. Endothelial cell-derived EVs can suppress monocyte activation due to the transfer of miRNAs such as miR-10a able to target several components of the nuclear factor-κB (NF-κB) pathway, including interleukin-1 receptor-associated kinase 4 [43]. Some studies have suggested that antigen-specific immunosuppressive EVs from autologous plasma may be used to inhibit inflammation. Interestingly, intraarticular injection of exosomes obtained from autologous conditioned serum was safe and reduced pain and inflammatory markers in RA patients who do not respond well to conventional therapy [44]. In addition, blood-derived exosomes may be negative regulators of osteoclast formation in RA [45].

Oxidative stress plays an important role in the regulation of the immune response in arthritis [46]. In RA patients there is a significant elevation of surface thiols on circulating monocytes while the newly released EVs of isolated CD14 + cells from these patients have decreased thiol levels and enhanced peroxyredoxin 1 expression compared with healthy subjects. These results suggest that production of EVs by human monocytes may regulate oxidative stress in these cells [47]. It has been reported that macrophages release EVs containing Gla-rich protein which is a calcification inhibitor in articular tissues and a possible anti-inflammatory agent in chondrocytes, synoviocytes and monocytes/macrophages. This protein may link inflammation and calcification events in the joint and is able to inhibit the production of pro-inflammatory cytokines in macrophages [48].

On the other hand, human neutrophils release EVs able to block inflammatory responses of macrophages and induce the release of transforming growth factor-β1 (TGF-β1) which can promote the resolution of the inflammatory response [49]. Neutrophil-derived microvesicles also exert chondroprotective actions *in vitro* and in murine models of inflammatory arthritis. It has been demonstrated that neutrophils migrate into inflamed joints to release microvesicles which penetrate into the cartilage. Neutrophil microvesicles require annexin A1 and its receptor formyl peptide receptor 2 to exert protective effects on chondrocytes which are mediated by TGF-β1 production, extracellular matrix deposition and inhibition of chondrocyte apoptosis. Interestingly, RA synovial fluids contain abundant neutrophil-derived microvesicles with a possible cartilage protecting role [50].

Dendritic cell (DC)-derived EVs deliver their content into the cytoplasm of acceptor DCs which could be a mechanism involved in fine-tuning of the immune response [51]. Nevertheless, These EVs are not only vehicles to deliver immunosuppressive factors from their parent cells as they exert antigen-specific effects which depend on the presence of molecules such as MHC class II and B7. Distal therapeutic effects were also observed after local administration of DC EVs suggesting that they may act by interacting with endogenous immune cells at the membrane level or by transfer of proteins and RNAs leading to an immunosuppressive and anti-inflammatory behavior of these cells [44]. Exosomes from immature DC may be partially immunosuppressive [52] and they can be modified to enhance this property. It has been suggested that IDO expression in DCs modifies exosomes to render them tolerogenic. Therefore, exosomes derived from DCs overexpressing indoleamine 2,3-dioxygenase (IDO) have an anti-inflammatory effect in collagen-induced arthritis (CIA) and delayed-type hypersensitivity murine models. These exosomes may directly interact with T cells and other antigen-presenting cells (APCs) to alter their function which was partially dependent on B7 costimulatory molecules [53]. In the CIA model, intravenous administration of exosomes derived from DCs expressing interleukin(IL)-10, DCs expressing IL-4 or DCs expressing FasL-effectively inhibited arthritis [54,55]. Injected exosomes are internalized by CD11c + cells at the site of injection and in the draining lymph node. Local administration of exosomes was also able to inhibit the inflammation of murine delayed-type hypersensitivity in both the treated and the untreated distal paws in a MHC class II dependent and MHC class I independent manner [55].

The ability of Treg cells to release exosomes is required to inhibit Th1 cell proliferation *in vivo* and prevent systemic disease. It has been reported that the miRNA content of exosomes play an important role in this inhibitory effect. Therefore, let-7d is transferred to Th1 cells and mediates the suppression of Th1 cell proliferation and interferon-γ (IFN-γ) secretion [56]. The transfer of the dominant negative form of inhibitor of NF-κB kinase 2 (IKK2) has been used to give rise to immature CD4 + CD25-Treg cells (dnIKK2-Treg). These cells release EVs containing specific miRNAs and inducible nitric oxide (NO) synthase which are delivered into target cells leading to block of cell cycle progression and induction of apoptosis. In addition, dnIKK2-Treg-EV-exposed T cells can be converted into regulatory cells [57].

Cell infiltration and the imbalance between cell proliferation and cell death contribute to pathological changes in RA. In particular, the resistance of synovial lymphocytes, macrophages and fibroblasts to apoptosis may play a role in the chronification of arthritis [58]. The bioactive death ligands FasL and APO2L/TRAIL are stored inside human T cells and secreted associated with EVs upon cell activation [59]. Interestingly, the number of EVs containing APO2L/TRAIL in synovial fluid is very low in RA patients and the persistence of activated T lymphocytes has been related to the resistance to Fas/CD95 and the inefficient secretion of EVs containing bioactive FasL and APO2L/TRAIL [60]. Therefore, EVs expressing APO2L/TRAIL may be a therapeutic approach for RA which has been explored in preclinical models. Intraarticular injection of artificial lipid vesicles resembling natural EVs with bound APO2L/TRAIL exerted anti-inflammatory effects and inhibited synovial hyperplasia in a model of antigen-induced arthritis in rabbits [61].

EVs from other sources may be of interest for their immunoregulatory properties. For instance, bovine milk contains EVs expressing CD63 and immunoregulatory miRNAs (miR-30a, -223, -92a). Oral administration of bovine milk derived EVs delayed the onset of CIA and diminished cartilage pathology, bone marrow inflammation and serum monocyte chemoattractant protein-1, IL-6 and anticollagen IgG2a levels, accompanied by reduced splenic Th1 (Tbet) and Th17 (RORγt) mRNA [62].

MSC EVs have been shown to reduce inflammation regulate immune responses and facilitate tissue regeneration [63]. There are complex interactions between MSCs and immune cells that may help to understand their immunomodulatory properties. The effects of MSCs are

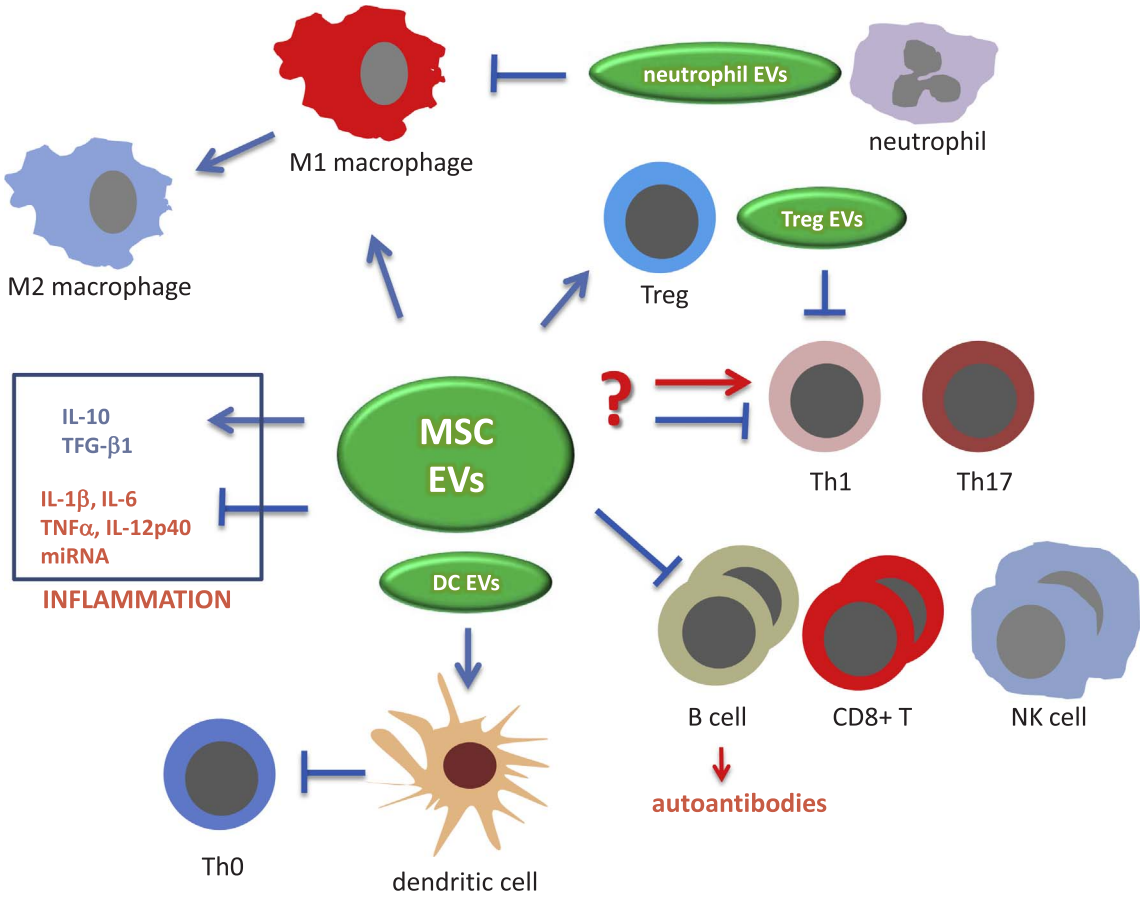

Fig. 1. Immunosuppressive effects of EVs from MSCs and other cell types. EVs from MSCs reduce the proliferation and differentiation of CD8+ T cells, B cells and NK cells while favor the differentiation of Treg cells and the switch of pro-inflammatory monocytes and macrophages (M1) to an anti-inflammatory phenotype (M2). The effect on CD4+ T cells has not been clearly demonstrated. Neutrophil EVs exhibit anti-inflammatory actions on macrophages. EVs from Treg cells inhibit Th1 cell proliferation. DC EVs can interact with T cells and APCs to alter their function. In inflammatory conditions, EVs inhibit the production of pro-inflammatory mediators and enhance that of anti-inflammatory and pro-resolution mediators in different cell types.

mediated by cell-to-cell contact and paracrine mechanisms due to the production of soluble molecules and EVs released into the extracellular milieu. The importance of cell-to-cell contact in immunosuppression by MSCs has been shown in different studies as well as the role of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 [64,65]. MSCs have been reported to secrete a wide range of molecules such as purines, bone morphogenetic proteins (BMPs), CD274, CCL2, connexin 43, IDO [66], prostaglandin E_2, [67] IL-6, IL-10, NO, [68] heme oxygenase-1, [69] tumor necrosis factor-inducible gene-6 (TSG-6), [70] leukemia inhibitory factor (LIF), CD95/CD95 ligand, galectins, human leukocyte antigen-G5 (HLA-G5), [71] and growth factors such as TGF-β1, [68] hepatic growth factor (HGF), [72] vascular endothelial growth factor (VEGF), platelet-derived growth factor, fibroblast growth factor (FGF), etc. [73]. Other ways of cellular communication between MSCs and immune cells include the bidirectional exchange of cytoplasmic components mediated by tunneling nanotubes derived from human T cells [74] and the transfer of EVs

Cellular therapy with stem cells showed a low engraftment and poor survival leading to the demonstration that MSCs act through paracrine effects in animal models of ischemic heart disease and acute kidney injury [75–79]. Therefore, it was demonstrated that only the fraction of the conditioned medium (CM) containing products > 1000 kDa (100–220 nm) provided cardioprotection in a mouse model of ischemia and reperfusion injury [80] leading to the confirmation that protective effects of MSC secretome depended on the presence of EVs [80–82]. In addition, EVs exert a modulating role on the effects of soluble mediators [83].

The beneficial effects of MSCs in glucocorticoid-refractory graft-versus-host disease in human patients have been related to immune response-modulating factors secreted by these cells and identified as EVs [84]. Similarly, EVs from umbilical cord (UC)-MSCs ameliorated the inflammatory immune reaction and kidney function in grade III-IV chronic kidney disease patients [85]. Phase I clinical studies with EVs have revealed a low toxicity and stability in plasma and different clinical studies have tested their potential in wound healing [86], hair regeneration [87], acne scars and skin rejuvenation [JSPH2012-082], type-1 diabetes [NCT02138331], the development of vaccines for different types of cancer or as vehicles for drug delivery to cells [88].

The degree of EV-mediated immunomodulation seems to be proportional to the ability of different immune cells to uptake these microparticles [89,90] leading to the inhibition of proliferation and differentiation processes [91]. EVs from MSCs may exert the strongest immunomodulatory effects on B cells compared with other lymphocyte subsets which may depend on the ability of B cells to incorporate EVs. Therefore, EVs from MSCs inhibit the proliferation of B cells and also of NK cells [89]. Nevertheless, the role on T cells has not been clearly demonstrated. It has been reported that exosomes from adipose-derived MSCs (AMSCs) exert an inhibitory effect on proliferation, differentiation and activation of T cells [92]. In contrast, microvesicles from bone marrow (BM)-MSCs have been shown to exert a lower immunomodulatory effect on T-cell proliferation compared with the parent cells [93]. Other reports failed to demonstrate any effect on lymphocyte proliferation by EVs from MSCs [74]. In another study, BM-MSC EVs exhibited *in vitro* immunomodulatory effects on T cells but they were different from those of their parent cells [94].

Immunosuppressive effects of BM-MSCs can be enhanced by priming with IFN-γ and tumor necrosis factor-α (TNFα) which leads to higher ICAM-1 expression and internalization of EVs by immune cells. In addition, primed EVs enhance the immunosuppressive ability of resting BM-MSCs towards T cells, which may be mediated by IDO increase [89]. Another report has indicated the possible contribution of cyclooxygenase-2 and different miRNAs to the immunosuppressive effects of cytokine-stimulated BM-MSCs [95]. Microvesicles and exosomes from murine MSCs have been shown to inhibit the proliferation of CD8+ T cells and the proliferation and activation of B cells. In addition, both types of EVs increased the Treg population but were without effect on CD4+ IFNγ+ T cells [96].

EVs released by MSCs are efficiently internalized by macrophages and induce proliferation and the transition of pro-inflammatory macrophages to an anti-inflammatory and pro-resolving M2 phenotype [97]. It was demonstrated that microvesicles from murine AMSCs were quickly incorporated into the intracytoplasmic region of M1-macrophages and promoted a M2-like phenotype and the reduction of pro-inflammatory miR-21 and miR-155. These results were confirmed *in vivo* in an experimental model of acute peritonitis [98]. Also, M2 polarization was induced by MSC EVs in mouse or human monocytes which in turn polarized activated CD4+ T cells to CD4+CD25+FoxP3+ Treg cells [99]. In contrast, EVs from UC-MSCs did not polarize monocytes [100]. Fig. 1 shows a summary of the immunosuppressive effects of EVs.

Few studies on the *in vivo* effects of EVs in arthritis models have been published. It has been reported that administration of EVs from murine MSCs ameliorated the symptoms in the mouse CIA model of RA [96]. In bovine serum albumin-induced synovitis in pigs, intraarticular administration of EVs from porcine BM-MSCs exerted anti-inflammatory effects with reductions in synovial lymphocytes count and TNFα expression. These EVs efficiently counteracted the antigen-driven T cell response and may represent a therapeutic strategy for the treatment of T cell mediated diseases such as RA [101].

Transfer of EVs components can play an important role in the effects of these microparticles. MSC EVs include a cargo of immunomodulatory proteins which may act in a synergistic manner [102]. These microparticles thus induce high levels of anti-inflammatory TGF-β1 and IL-10 [103,104], and inhibit pro-inflammatory IL-1β, IL-6, TNF-α and IL-12p40 [99]. Microvesicles derived from mouse BM-MSCs express regulatory molecules present in parent cells such as programmed death-ligand 1 (PD-L1), galectin-1 (Gal-1) and TGF-β1 which confer tolerogenic properties to these microparticles [105]. Interestingly, PD-L1 contributes to the development of inducible T regulatory (iTreg) cells [106] while Gal-1 has been shown to induce growth arrest and apoptosis of activated T cells and contribute to the promotion and generation of Treg cells [107,108]. Therefore, Gal-1 gene therapy or protein administration to DBA/1 mice inhibited clinical and histological manifestations of arthritis in the CIA model [109]. The results of these studies support the interest of EVs in the treatment of chronic inflammatory and autoimmune disorders. In addition, microvesicles from MSCs contain ribonucleoproteins involved in the intracellular traffic of RNAs as well as selective miRNAs which may be transferred to target cells [110] and likely exert immunomodulatory effects in arthritic diseases (reviewed in [111]). Apart from the presence and properties of endogenous miRNAs, loading of EVs with miRNAs or anti-miRs may be an interesting approach to enhance the immunoregulatory activity of these microparticles in chronic inflammatory conditions [42].

4. Regenerative properties of extracellular vesicles in joint conditions

Joint conditions are important targets of MSC therapy mainly to treat chondral and/or bone lesions and defects resulting from injury or trauma, or in OA. In the last years, the possible applications of MSCs in cartilage repair used alone or combined with biomaterials have been extensively explored. MSCs are injected into the joint space, or implanted in a scaffold matrix or as tissue engineered constructs in order to create a favorable microenvironment for tissue repair (for review see Ref. [112]). Stem cells are capable of selectively homing to injured tissues and differentiating into several types of cells to repair the lesion and improve the affected function. Humoral mediators produced by injured tissue would be chemotactic for stem cells, they also stimulate local proliferation of endogenous or exogenous stem cells or could be a signaling mechanism to expand the pool of bone marrow progenitor cells in response to tissue injury [113]. Nevertheless, it has been reported that chondrogenesis in 3D culture generates constructs whose mechanical properties are inferior to constructs formed with chondrocytes [114] leading to studies on different strategies to improve the chondrogenic potential of MSCs [115].

Cell differentiation and engraftment would not be the sole mechanisms for tissue regeneration as transplanted cells become fewer and disappear soon after transplantation [115]. Additionally, MSCs exhibit a variety of trophic activities relevant to musculoskeletal therapy and can promote chondrogenesis, osteogenesis, musculogenesis, tenogenesis, angiogenesis and neurogenesis (reviewed in [73]). The efficacy of MSC therapies in joint repair has been demonstrated in many animal models and clinical studies [116–126].

The effectiveness of many MSC-based therapies in tissue repair has been attributed to the paracrine secretion of these cells as only a small percentage of the MSC populations injected into the joint actually remain at the site of injury (reviewed in [8]). The MSC secretome would promote tissue repair by modulating the local microenvironment and supporting growth and activity of local cells. Nevertheless, the composition of MSC secretome is quite complex and varies depending on the microenvironment of cells [127]. For instance, MSCs have a differential response to synovial fluid from early- versus late-stage OA, with a higher secretion of CXCL8, IL6 and CCL2 in the first case [128]. Accordingly, pretreatment of MSCs with different factors can improve the release of immunomodulating or regenerating mediators [129] as it has been shown by priming the parent MSCs with lipopolysaccharide [130].

The CM of MSCs contains a wide range of cytokines, chemokines, hormones, lipid mediators, cytokines, growth factors and extracellular matrix components which can mediate tissue healing. The regenerative properties of CM from MSCs have been explored in many different tissues (reviewed in [88]). Therefore, MSC CM can regenerate bone through mobilization of endogenous stem cells, angiogenesis and osteogenesis [131] and promote periodontal tissue regeneration [132] and healing of bisphosphonate-related osteonecrosis of the jaw in rats [133]. Interestingly, the therapeutic efficacy of human BM-MSCs CM was demonstrated in a human clinical study. This CM containing insulin-like growth factor-1, VEGF, TGF-β1 and HGF, in beta-tricalcium phosphate or an atelocollagen sponge, regenerated alveolar bone [134].

Treatment of OA chondrocytes or synovial cells with CM from BM-MSCs or AMSCs in an inflammatory milieu inhibits the production of inflammatory and catabolic agents [9,10]. In this respect, we have reported that CM from AMSCs exert protective effects in OA chondrocytes [20,21]. Therefore, factors produced by MSCs can enhance the anabolic properties of a wide range of cells such as chondrocytes, chondrocyte progenitor cells, cartilage-derived stem/progenitor cells, synovium-resident multipotent progenitor cells, osteoblasts/osteoclasts/resident MSCs in subchondral bone and chondrogenic cells within the infrapatellar fat pad [73].

The EVs present in CM show a great potential in the regeneration of joint tissues to replace stem cell-based therapy. EVs secreted by hMSC carry hyaluronan on their surface which is able to interact with proteins and proteoglycans of extracellular matrix to maintain tissue homeostasis, and contribute to extracellular matrix remodeling and tissue healing [135,136]. EVs express adhesion molecules to bind to and interact with cells, but they are also able to bind to extracellular matrix components. EVs from some cell types contain extracellular matrix

regulatory proteins involved in re-structuring, cytokine release, angiogenesis and cell migration as well as lysyl oxidases which crosslink collagens and elastin [137,138]. In particular, exogenous lysyl oxidases have been shown to be useful in cartilage integration problems [139]. Interestingly, EVs from different cell types are able to transfer the mRNA of growth factors and their receptors to tissue cells to initiate tissue repair responses [140,141].

Treatments with MSC EVs are able to reproduce the main actions of CM suggesting that these microparticles are relevant mediators. EVs from naïve or genetically modified MSCs may be used to improve the regenerative properties of these cells as they can modulate the microenvironment of damaged cartilage to promote repair or to enhance the chondrogenic ability of these cells [124]. Different scaffolds have been investigated to retain MSC EVs and promote cartilage repair. One of them is a photoinduced imine crosslinking hydrogel glue with excellent biocompatibility and integration with cartilage matrix which has been tested to prepare an acellular tissue patch for cartilage regeneration [142].

Interestingly, OA chondrocytes internalize EVs from BM-MSCs leading to the upregulation of aggrecan and type II collagen. In addition, gene expression of IL-1, IL-6, IL-8 and IL-17 as well as collagenase activity induced by TNFα were significantly reduced [143]. It has also been reported that EVs from mouse BM-MSC exert anti-apoptotic effects in chondrocytes and immunosuppressive effects in macrophages. *In vivo* administration of these EVs partly protected cartilage and bone in the murine collagenase model of OA [144]. We have provided evidence that microvesicles and exosomes from human AMSC CM exert anti-inflammatory and protective effects in OA osteoblasts [145] and chondrocytes (our unpublished results) *in vitro*. Anti-inflammatory and chondroprotective effects of EVs derived from AMSCs have also been described in murine cells [146].

The release of pro-inflammatory mediators and reactive oxygen species can result in mitochondrial changes, inflammation, oxidative stress and DNA alterations which can induce premature senescence [37]. We have recently shown that microvesicles and exosomes from human AMSCs reduce the production of inflammatory mediators, mitochondrial membrane alterations and oxidative stress in OA osteoblasts which results in the down-regulation of cell senescence [145] (Fig. 2).

Different studies have demonstrated that EVs enhance skeletal muscle [147], bone [148] and cartilage [142] regeneration. Administration of AMSC EVs in a model of skeletal muscle injury reduced the inflammatory response and accelerated the muscle regeneration process [97]. BM-MSC-derived EVs led to bone formation in calvarial bone defects with an essential role for miR-196a in the regulation of osteoblastic differentiation [148]. Injection of exosomes from human

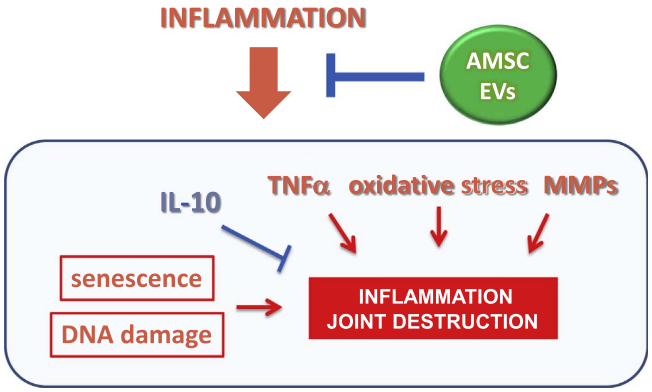

Fig. 2. Joint protective effects of AMSC EVs. In OA osteoblasts or chondrocytes subjected to inflammatory conditions, EVs reduce the production of pro-inflammatory and catabolic mediators as well as the induction of DNA damage and cell senescence while the production of the anti-inflammatory cytokine IL-10 is enhanced.

induced pluripotent stem cell-derived MSCs (iPS-MSCs) by intravenous route prevented osteonecrosis induced by steroid in rats. This treatment activated the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway on endothelial cells leading to local angiogenesis [149]. Protective effects of these type of exosomes were also observed in an ovariectomized rat model [150] and, in combination with a tricalcium phosphate scaffold, in rat calvarial bone defect. *In vitro* studies showed that these exosomes can be internalized into BM-MSCs and enhanced the proliferation, migration, and osteogenic differentiation of these cells. Activation of the PI3K/Akt signaling pathway by exosomes likely plays an important role in these effects [151].

Intraarticular injection of exosomes obtained from human ESCs has been shown to completely restore cartilage and subchondral bone in a model of osteochondral defects in rats [152]. In another study, exosomes secreted by human synovial MSCs were internalized by human chondrocytes *in vitro* and induced proliferation and migration but reduced extracellular matrix production. These effects were due to the high Wnt5a and Wnt5b expression in these cells which activated YAP and led to the suppression of SOX9 expression. *In vivo* intraarticular injection of exosomes resulted in a weak protective effect in the rat OA model established by transecting the medial collateral ligament and the medial meniscus [153]. In another OA model in mice injected with collagenase, intraarticular administration of human synovial MSC exosomes significantly attenuated OA progression. In the same model, injection of exosomes from human iPS-MSCs had a superior therapeutic effect. Therefore, these cells may be a better source of exosomes for cartilage repair with other advantages as they can be induced from patient-specific adult somatic cells such as peripheral blood cells without an invasive harvesting and with a high yield. In addition, autologous cells can be used overcoming ethical issues and immune activation [154].

Some treatments using EVs from other sources may also be useful in OA. As an example, EVs from autologous conditioned serum have been shown to protect human OA cartilage from glycosaminoglycan loss in basal conditions and in the presence of IL-1β [155]. There is an ongoing observational study that evaluates the characteristics of autologous platelet-rich plasma (PRP) in the therapy and treatment of musculoskeletal pain and OA. This study will test the hypothesis that PRP characteristics, such as platelet and microparticle content and composition can be predictive for clinical outcome for PRP treatments (NCT02726464).

Further studies are necessary to establish the mechanisms underlying the regenerative effects of EVs but these microparticles contain many regulatory molecules that may be transferred to target cells and contribute to their biological effects. It has been suggested that EVs delivery to damaged tissue may contribute to epigenetic reprogramming of target cells [156,157]. MSC EVs repair ability can depend on the restoration of cartilage homeostasis. In OA, there is chondrocyte loss or cellular senescence induced by abnormal mechanical stress, inflammation, oxidative stress and mitochondrial dysfunction [158,159]. EVs may transfer to chondrocytes glycolytic enzymes such as phosphoglucokinase and pyruvate kinase, and ATP generating enzymes such as adenylate kinase and nucleoside-diphosphate kinase that may compensate the reduced mitochondrial ATP production in OA chondrocytes. In addition, MSC EVs contain CD73 which is able to convert the extracellular ATP released by injured tissues to adenosine. It is known that EVs induce cell proliferation through adenosine-mediated phosphorylation of extracellular signal-regulated kinase (ERK)-1/2 and Akt [160].

On the other hand, the regulation of immune cells and the pro-inflammatory environment plays an important role in tissue regeneration. M1 polarized synovial macrophages from OA patients secrete factors inhibiting MSC chondrogenesis [161] whereas MSCs can induce M2 polarization which reduce inflammation and improve cartilaginous tissue regeneration [162]. As EVs can exert immunomodulatory and anti-inflammatory effects, it is likely that these properties contribute to

joint protection and repair.

MSC derived EVs contain a wide range of miRNAs which may facilitate intercellular communication and contribute to different phases of the healing process [97,163] . Many of these miRNAs are involved in signal transduction, cartilage metabolism and OA progression. For instance, mir-23b is a mediator of chondrocyte differentiation of human MSCs [164] and miR-221 and miR-92a may contribute to the regulation of cell proliferation and differentiation of chondroprogenitor cells [165–167] . It is also known that key catabolic enzymes such as aggrecanase-2 and metalloproteinase-13 are the target of miR-125b and miR-320, respectively [168,169] . The expression of miR-320 is reduced in OA cartilage compared with normal cartilage. This miRNA regulates chondrogenesis and IL-1β-stimulated catabolic effects in mouse chondrocytes [169] . Another miRNA with therapeutic potential in OA is miR-140, which regulates cartilage homeostasis and development [170] . Interestingly, modification of synovial MSCs to overexpress miR-140-5p improved EV properties *in vitro* and *in vivo* leading to a significant inhibition of cartilage degradation in a surgical model of knee OA in rats [153]. All together these data support the interest of EVs containing miRNAs with beneficial effects on joint metabolism to develop potential therapeutic approaches in OA

5. Extracellular vesicles in the physiopathology of joint conditions

EVs mediate cell communication in pathological states and may act as signaling structures involved in the induction and amplification of immunity and inflammation. Therefore, some EVs can play a pathogenic role in joint conditions (for extensive reviews see Refs. [171–176]). EVs may exert different roles in inflammation depending on the cell source, cell target and the environment where they can be influenced by multiple factors. Some studies have revealed that EVs are a component for autocrine and/or paracrine stimulation although they may also initiate counter-regulatory mechanisms which potentially contribute to the resolution of inflammation. In this context, EVs released by activated human monocytes have been shown to activate NF-κB and production of cytokines and oxygen radicals which may lead to the amplification of the inflammatory response. At the same time, these EVs are able to enhance PPAR-γ expression which is involved in the control of inflammation [177]. It is also known that EVs from T cells can modulate the effects of TNFα, suggesting a cross-talk between cytokines and EVs. Besides promoting the induction of inflammation, treatment with EVs significantly upregulated a number of anti-inflammatory genes [83].

The stimulation of immune responses by EVs in certain situations can initiate or exacerbate autoimmune diseases. EVs may exert immunostimulatory effects by a number of mechanisms. EVs express different molecules of the parent cells and may transfer antigens, MHC molecules and costimulatory molecules to immune cells. As an example, DCs produce EV-associated MHC class I complexes which are transferred to other naive DCs for efficient CD8+ T cell priming which can be viewed as an amplification process for DC-mediated CTL responses [178]. Similarly, EVs from both human and murine B lymphocytes are able to induce antigen-specific MHC class II-restricted T cell responses. These studies support the view that EVs produced by APCs may act as vehicles for MHC class II-peptide complexes involved in maintenance of long-term T cell memory or T cell tolerance [179].

Synovial EVs formed in an inflammatory environment may stimulate articular cells to release more inflammatory mediators and degradative enzymes and thus contribute to articular damage [27,180]. Some EVs can also contain degradative enzymes and therefore EVs released by rheumatoid synovial fibroblasts degrade aggrecan in a tissue inhibitor of metalloproteinase-3-sensitive manner which may facilitate cell invasion through aggrecan-rich extracellular matrices [181]. Similarly, hexosaminidase activity is found to be associated with RA synovial fibroblast-derived EVs [180]. On the other hand, it has been reported that EVs released by chondrocytes contribute to pathologic mineralization of cartilage in musculoskeletal pathologies such as OA [182] and their secretion is regulated by autophagy [183].

More studies are necessary to dissect EV signaling pathways and molecular mechanisms in the physiopathology of joint conditions. As EVs produced by some cell types may be mediators of the pathophysiological changes that occur in the joint environment, a therapeutic strategy may be the inhibition of production and release, modification of harmful content or elimination of microparticles contributing to pathological processes [184].

6. Extracellular vesicles as biomarkers of joint disease

There is a considerable interest in identifying noninvasive specific biomarkers which may reflect the alterations in joint tissues. At present, prognostic tools especially for OA and spondyloarthritis are still lacking. Early identification of predictive markers is crucial to address the risk, the presence, the evolution and the response to treatment in chronic joint conditions [185]. The release of EVs into the extracellular space allows to examine them in body fluids as novel candidates for disease biomarkers to use in diagnosis, prognosis and treatment. Of note, in situations where the same biomarker molecules can be indicative of more than one condition, EVs would be the method of choice to trace the cell type causing the alteration. These microparticles can be immuno-isolated based on recognition of a significantly enriched protein on the membrane surface [186].

Serum EVs are enhanced in RA. In particular, endothelial EVs have a deleterious effect on endothelial cell function and may be a marker of vascular damage [187] while platelet-derived EVs levels may be related to disease activity [188]. Nevertheless, circulating EVs exposing complement components, C reactive protein or serum amyloid-P are elevated in early active RA although effective drug treatments do not decrease their levels suggesting a limited value as biomarkers [189].

Other reports indicate that serum EVs may be useful as additional markers of disease activity in patients with RA. For instance, differences in EV levels of amyloid A and lymphatic vessel endothelial hyaluronic acid receptor-1 have been found between the clinical remission and non-clinical remission groups [190]. In addition, high expression of Hotair has been demonstrated in blood mononuclear cells and serum EVs of RA patients whereas a lower level of Hotair was detected in differentiated osteoclasts and rheumatoid synoviocytes [191]. Platelet EVs are also elevated in RA and other inflammatory arthritis synovial fluid compared with OA and may play a role in the amplification of the inflammatory process. In this respect, collagen receptor glycoprotein VI has been identified as a key trigger for platelet EV generation in arthritis [192].

Synovial EVs contain citrullinated proteins, which are known autoantigens and biomarkers in RA [193]. In synovial fluid from RA patients, the number of microvesicles positive for receptor activator of NF-κB and its ligand are increased as well as CD3+ and CD8+ microvesicles which might reflect a locally enhanced activation of CD8+ T cells [21]. In addition, CD4+ T-cell-derived CD161+ CD39+ and CD39+ CD73+ EVs in synovial fluid have been recently proposed as reciprocal biomarkers for RA [194].

Differences in miRNA expression in EVs may lead to propose new biomarkers in joint conditions [174,195]. In the last years many studies have focused on circulating miRNAs as biomarkers of disease which represent an important part of EV composition (reviewed in [175]). The changes in synovial fluid-derived EV miRNA with joint alterations provide a unique opportunity to discover candidate biomarkers. Interestingly, studies of miRNA expression in synovial fluid EVs from OA patients have shown sex specific changes. Therefore, in female patients, miR16-2-3p was upregulated and miR26a-5p, miR146a-5p and miR-6821-5p were downregulated while in male patients, miR-6878-3p was downregulated and miR-210-5p was upregulated. These results also suggested that estrogen might play an important role in EV derived miRNA [196]. Therefore, a gender dimension should be considered in

the investigation of specific biomarkers for joint conditions.

7. Challenges in EV research

Several nomenclature and methodological challenges have raised concerns among the community about the reproducibility and comparability of the different reports published in recent years. In particular, the disparity of isolation and characterization approaches, and the lack of unified nomenclature and handling criteria are hindering the understanding of EVs biological functions [18]. In that regard, organizations such as the International Society for Extracellular Vesicles (ISEV) have published guidelines in an increasing effort to integrate the currently accepted isolation and characterization methods [18,197].

Vesicles shed from the cell plasma membrane are often called microvesicles, microparticles or ectosomes, with a sized ranged between 50 and 100 nm to even few micrometers depending on the author's criteria and the isolation method. Small vesicles secreted from multivesicular endosomal bodies are usually called exosomes and classically considered to be under 150 nm in diameter, but most common isolation procedures based on the use of 200-nm pore filters and ultracentrifugation, isolate mixed EV populations. In practice, EV classification is not clear-cut as microparticles exhibit overlapping similarities in size, morphology, density and protein markers of both endosome and plasma membrane [16] and even the presence of different subpopulations within the same EVs class has been demonstrated [17,22]. In addition, a single cell can release EVs with differences in size, biogenesis and content which can vary depending on the cell type and its physiologic state [198].

From upstream sample handling to isolation and characterization, there is presently no single standardized method to universally obtain pure EV products. Generally, a highly pure EV isolate is obtained at the expense of therapeutic potency, yield, cost and/or scalability. These considerations are of critical importance when dealing with EVs as therapeutic agents, as industrial scale production must deliver an acceptable compromise between purity, activity and cost [199]. Currently, characterization efforts have focused on physical properties such as size and concentration, and vesicular content in terms of protein, lipid and nucleic acid composition. As the smallest EVs reach sizes of 50 nm or even less, current size analysis methods struggle to reach this detection limit, making comparisons of different concentrations difficult and statistically compromised.

Vesicular cargo includes proteins, RNA, DNA, lipids and metabolites, and may be inside EVs or on their surface. Subvesicular localization must be considered during characterization procedures to avoid artefacts and false positives. Importantly, the isolation method severely impacts the purity of EVs and therefore the omics profiles [200] and possible EV applications. However, as EV isolates contain disparate populations, current data should be considered as an average of the RNA content of all EV subpopulations. Additionally, most biofluids contain potential contaminants such as RNA- and miRNA-carrying proteins, making analyses difficult to decipher even after treatment with RNAses [201].

It is crucial to improve the methods to isolate and characterize the different EV types. This issue is a source of confusion leading to contradictory results but it is also the first necessary step for studies of pharmacological activity and therapeutic efficacy. As many factors can influence the reproducibility of effects, different steps need to be taken to assure homogenous EV preparations and guarantee their efficacy and safety. In the last years, the International Society for Extracellular Vesicles (ISEV) has released position papers and the Minimal Information for Studies on EVs (MISEV) to help researchers overcome these problems. Furthermore, to increase reproducibility and transparency of EV methodologies, the EV-TRACK knowledgebase has been recently developed [202]. Strict standardized protocols must be implemented to effectively control all aspects of EV production and application, from culture of source cells to medicinal product preparation and administration. Qualitative and quantitative EV technologies need to be thoroughly validated. New technologies may help to advance this research field. For instance, EV uptake can be determined at single cell level using the Cre reporter methodology or bioluminescence methods can be employed to determine EV release and uptake and new-omics approaches have been incorporated to improve the knowledge of molecular EV components [30]. In addition, there is a need for normalization and control in sample collection and methods for keeping and transporting EV samples. All these points are essential to detect relevant differences between health and disease in clinical studies. It is not surprising that results found in the literature showed EV clinical studies of small populations with small portion of large effect size. Improved methodologies and study design are needed including larger numbers of samples in order to determine whether there is an effect at the population level [203].

Human EV-based therapeutics is subjected to the regulatory frameworks of biological medicinal products covering preclinical development, quality aspects, non-clinical safety requirements and the clinical testing. In the context of EV-related therapies and their approval, a complete *in vitro* and *in vivo* testing must be outlined. This should at least include assays to identify and characterize the components of the EV isolate (molecular fingerprinting), potency assays to quantify the EV-mediated therapeutic effect, and functional tests to determine their mechanisms of action as well as pharmacokinetic and toxicology studies. In the particular case of EV research, issues such as localization of molecules —inside the vesicle, embedded in the membrane, or associated outside— and mechanisms of cell-EV interaction —mainly vesicle internalization or plasma membrane receptor signaling— must be carefully taken into consideration for a thorough pharmacological validation [199].

For clinical application, compliance with safety standards related to inadvertent microbial and viral contamination and GxP standards (Good Manufacturing/Good Laboratory/Good Distribution/Good Clinical/Good Scientific Practice or GMP/GLP/GDP/GCP/GSP) is necessary for the production and quality control [1]. As a further step, one important hurdle is the ability to produce consistent products on a large scale. There is a need of developing clinical-grade robust and stable manufacturing processes.

8. Perspectives

Interest in cell-derived EVs has exponentially increased due to their proposed contribution to homeostasis and disease, and their potential as future therapeutic and diagnostic tools. In particular, EVs have recently received a great deal of attention as a possible better alternative to MSC therapy in autoimmune conditions and tissue regeneration. The induction of immunological reset by MSC EVs has become an attractive possibility in RA and other autoimmune conditions. while the use of EVs for joint repair and OA could potentially be a better cost-effective therapy compared with MSC administration [160].

EVs offer the possibility to develop cell-free therapeutic approaches with less regulatory obstacles and clinical risks associated to cell therapies. Besides, they may have potential advantages in biomanufacturing, storing and distribution and may represent a more reproducible therapeutic tool [90,94]. EVs contain many biomolecules from the parent cells and can have advantages compared with cell therapy, as injected cells may die or fail to fully home into the lesion while EVs injection allows for a more precise dosing schedule and a better control of treatment or suspension of administration. The use of EVs may also eliminate problems such as blood vessels occlusion and generation of altered cell phenotypes [160,204].

Compared with MSCs, which produce different molecules according to the microenvironment leading to complex interactions or can exhibit opposite effects depending on the stimulus used to trigger immune cells [205], EVs may lead to results less dependent on the environment and more predictable. The content of these microparticles is protected from

enzyme degradation, and this natural mechanism can be used to deliver active molecules to cells. In this respect, EVs are less likely to alter target cells than artificial nanoparticles. The small size may be an advantage in relation with the selection of administration routes in comparison with cell therapy. In addition, their bi-lipid layer vesicular structure is membrane permeable and their surface proteins may confer targeting ability due to their affinity for specific cell membranes or extracellular matrix in diseased tissues [79,157,206].

EVs have a lower immunogenic potential compared with cells [204] and thus allogeneic EVs have been reported to be safe and may be an appropriate source for large-scale production [160] in preclinical studies and clinical applications. In this respect, technological advances can improve large-scale preparation of EVs. For instance, recent studies have demonstrated that microvesicle production by MSCs can be amplified using a 3-D bioprocessing method keeping the biological activity of these microparticles [207].

Modification of EVs may improve their properties to regulate different processes. Therefore, the parent cells could be primed or genetically modified and then expanded in order to produce modified EVs e.g. without histocompatibility antigens to minimize the possibility of immune reactions, expressing relevant proteins, lipids or RNA to maximize the pharmacological effects, or molecules that facilitate their tropism and retention in damaged tissues or recognition by target cells thus improving treatment selectivity. In addition, different approaches e.g. integration in a hydrogel-scaffold or chondroitin sulfate sponge are in development to facilitate stable long-term delivery to joint tissues [174].

On the other hand, the determination of EVs can be useful as biomarkers of joint diseases as the content of microparticles is related to the parent cell and its microenvironment. In this respect, miRNA and proteome analyses represent promising approaches.

The standardization of isolation and characterization methods is crucial for the development of this novel tool. It is apparent that much work both *in vitro* and *in vivo* is needed in order to better understand the biogenesis, composition, appropriate delivery technique, *in vivo* stability and distribution, internalization, mechanisms of action, efficacy, long-term actions and safety of EVs.

Although we only focus on limited aspects of EVs, there are new mechanisms to be identified which may lead to other potential applications of these microparticles. Taken as a whole, the studies outlined in this review reinforce the increasing interest in the field and the efforts devoted to understand EV biology. Nevertheless, the complexity of the topic has raised a number of important questions which need to be answered before this novel approach can progress to clinical applications in joint conditions.

Acknowledgements

This work has been funded by grants SAF2013-48724R (MINECO,FEDER, Spain) and PROMETEOII/2014/071 (Generalitat Valenciana, Spain).

Conflict of interests

There is no conflict of interest to declare.

References

[1] T. Lener, M. Gimona, L. Aigner, V. Borger, E. Buzas, G. Camussi, N. Chaput, D. Chatterjee, F.A. Court, H.A. Del Portillo, L. O'Driscoll, S. Fais, J.M. Falcon-Perez, U. Felderhoff-Mueser, L. Fraile, Y.S. Gho, A. Gorgens, R.C. Gupta, A. Hendrix, D.M. Hermann, A.F. Hill, F. Hochberg, P.A. Horn, K.D. de, L. Kordelas, B.W. Kramer, E.M. Kramer-Albers, S. Laner-Plamberger, S. Laitinen, T. Leonardi, M.J. Lorenowicz, S.K. Lim, J. Lotvall, C.A. Maguire, A. Marcilla, I. Nazarenko, T. Ochiya, T. Patel, S. Pedersen, G. Pocsfalvi, S. Pluchino, P. Quesenberry, I.G. Reischl, F.J. Rivera, R. Sanzenbacher, K. Schallmoser, I. Slaper-Cortenbach, D. Strunk, T. Tonn, P. Vader, B.W. van Balkom, M. Wauben, S.E. Andaloussi, C. Thery, E. Rohde, B. Giebel, Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper, J. Extracell. Vesicles 4 (2015) 30087.
[2] G. Raposo, W. Stoorvogel, Extracellular vesicles: exosomes, microvesicles, and friends, J. Cell Biol. 200 (2013) 373–383.
[3] H. Kalra, R.J. Simpson, H. Ji, E. Aikawa, P. Altevogt, P. Askenase, V.C. Bond, F.E. Borrás, X. Breakefield, V. Budnik, Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation, PLoS Biol. 10 (2012).
[4] M. Simons, G. Raposo, Exosomes-vesicular carriers for intercellular communication, Curr. Opin. Cell. Biol. 21 (2009) 575–581.
[5] A. Bobrie, M. Colombo, G. Raposo, C. Théry, Exosome secretion: molecular mechanisms and roles in immune responses, Traffic 12 (2011) 1659–1668.
[6] G.S. Firestein, Evolving concepts of rheumatoid arthritis, Nature 423 (2003) 356–361.
[7] R. Agca, S.C. Heslinga, S. Rollefstad, M. Heslinga, I.B. McInnes, M.J. Peters, T.K. Kvien, M. Dougados, H. Radner, F. Atzeni, J. Primdahl, A. Sodergren, J.S. Wallberg, R.J. van, C. Zabalan, T.R. Pedersen, L. Jacobsson, V.K. de, M.A. Gonzalez-Gay, A.G. Semb, G.D. Kitas, Y.M. Smulders, Z. Szekanecz, N. Sattar, D.P. Symmons, M.T. Nurmohamed, EULAR recommendations for cardiovascular disease risk management in patients with rheumatoid arthritis and other forms of inflammatory joint disorders: 2015/2016 update, Ann. Rheum. Dis. 76 (2017) 17–28.
[8] P.A. Reilly, J.A. Cosh, P.J. Maddison, J.J. Rasker, A.J. Silman, Mortality and survival in rheumatoid arthritis: a 25 year prospective study of 100 patients, Ann. Rheum. Dis. 49 (1990) 363–369.
[9] I.B. McInnes, G. Schett, Pathogenetic insights from the treatment of rheumatoid arthritis, The Lancet 389 (2017) 2328–2337.
[10] M.B. Goldring, M. Otero, Inflammation in osteoarthritis, Curr. Opin. Rheumatol. 23 (2011) 471–478.
[11] S.B. Abramson, M. Attur, Developments in the scientific understanding of osteoarthritis, Arthritis Res. Ther. 11 (2009) 227.
[12] J.P.G. Sluijter, S.M. Davidson, C.M. Boulanger, E.I. Buzas, D.P.V. de Kleijn, F.B. Engel, Z. Giricz, D.J. Hausenloy, R. Kishore, S. Lecour, J. Leor, R. Madonna, C. Perrino, F. Prunier, S. Sahoo, R.M. Schiffelers, R. Schulz, L.W. Van Laake, K. Ytrehus, P. Ferdinandy, Extracellular vesicles in diagnostics and therapy of the ischaemic heart: Position Paper from the Working Group on Cellular Biology of the Heart of the European Society of Cardiology, Cardiovasc. Res. 114 (2018) 19–34.
[13] R. Lacroix, C. Judicone, M. Mooberry, M. Boucekine, N.S. Key, F. Dignat-George, Standardization of pre-analytical variables in plasma microparticle determination: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop, J. Thromb. Haemost. 11 (2013) 1190–1193.
[14] A.J. Mitchell, W.D. Gray, S.S. Hayek, Y.A. Ko, S. Thomas, K. Rooney, M. Awad, J.D. Roback, A. Quyyumi, C.D. Searles, Platelets confound the measurement of extracellular miRNA in archived plasma, Sci. Rep. 6 (2016) 32651.
[15] G.V. Shelke, C. Lasser, Y.S. Gho, J. Lotvall, Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum, J. Extracell. Vesicles 3 (2014) 24783.
[16] D.D. Taylor, S. Shah, Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes, Methods 87 (2015) 3–10.
[17] E. Willms, H.J. Johansson, I. Mager, Y. Lee, K.E. Blomberg, M. Sadik, A. Alaarg, C.I. Smith, J. Lehtio, A.S. El, M.J. Wood, P. Vader, Cells release subpopulations of exosomes with distinct molecular and biological properties, Sci. Rep. 6 (2016) 22519.
[18] F.A.W. Coumans, A.R. Brisson, E.I. Buzas, F. Dignat-George, E.E.E. Drees, S. El-Andaloussi, C. Emanueli, A. Gasecka, A. Hendrix, A.F. Hill, R. Lacroix, Y. Lee, T.G. van Leeuwen, N. Mackman, I. Mager, J.P. Nolan, E. van der Pol, D.M. Pegtel, S. Sahoo, P.R.M. Siljander, G. Sturk, W.O. De, R. Nieuwland, Methodological Guidelines to Study Extracellular Vesicles, Circ. Res. 120 (2017) 1632–1648.
[19] R. Vogel, F.A. Coumans, R.G. Maltesen, A.N. Boing, K.E. Bonnington, M.L. Broekman, M.F. Broom, E.I. Buzas, G. Christiansen, N. Hajji, S.R. Kristensen, M.J. Kuehn, S.M. Lund, S.L. Maas, R. Nieuwland, X. Osteikoetxea, R. Schnoor, B.J. Scicluna, M. Shambrook, V.J. de, S.I. Mann, A.F. Hill, S. Pedersen, A standardized method to determine the concentration of extracellular vesicles using tunable resistive pulse sensing, J. Extracell. Vesicles 5 (2016) 31242.
[20] X. Osteikoetxea, A. Balogh, K. Szabo-Taylor, A. Nemeth, T.G. Szabo, K. Paloczi, B. Sodar, A. Kittel, B. Gyorgy, E. Pallinger, J. Matko, E.I. Buzas, Improved characterization of EV preparations based on protein to lipid ratio and lipid properties, PLoS One 10 (2015) e0121184.
[21] B. Gyorgy, T.G. Szabo, L. Turiak, M. Wright, P. Herczeg, Z. Ledeczi, A. Kittel, A. Polgar, K. Toth, B. Derfalvi, G. Zelenak, I. Borocz, B. Carr, G. Nagy, K. Vekey, S. Gay, A. Falus, E.I. Buzas, Improved flow cytometric assessment reveals distinct microvesicle (cell-derived microparticle) signatures in joint diseases, PLoS One 7 (2012) e49726.
[22] J. Kowal, G. Arras, M. Colombo, M. Jouve, J.P. Morath, B. Primdal-Bengtson, F. Dingli, D. Loew, M. Tkach, Th+®ry C. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes, PNAS 113 (2016) E968–E977.
[23] P. van Pham, Concise review: extracellular vesicles from mesenchymal stem cells as cellular therapy, Biomed. Res. Ther. 4 (2017) 1562–1573.
[24] M. Durcin, A. Fleury, E. Taillebois, G. Hilairet, Z. Krupova, C. Henry, S. Truchet, M. Trötzmüller, H. Köfeler, G. Mabilleau, O. Hue, R. Andriantsitohaina, P. Martin, S. Le Lay, Characterisation of adipocyte-derived extracellular vesicle subtypes identifies distinct protein and lipid signatures for large and small extracellular vesicles, J. Extracell. Vesicles 6 (2017) 1305677.
[25] S. Mathivanan, H. Ji, R.J. Simpson, Exosomes: extracellular organelles important in intercellular communication, J. Proteomics 73 (2010) 1907–1920.
[26] M. Eldh, K. Ekstrom, H. Valadi, M. Sjostrand, B. Olsson, M. Jernas, J. Lotvall, Exosomes communicate protective messages during oxidative stress; possible role

of exosomal shuttle RNA, PLoS ONE 5 (2010) e15353.
[27] T. Kato, S. Miyaki, H. Ishitobi, Y. Nakamura, T. Nakasa, M.K. Lotz, M. Ochi, Exosomes from IL-1beta stimulated synovial fibroblasts induce osteoarthritic changes in articular chondrocytes, Arthritis Res. Ther. 16 (2014) R163.
[28] N. Iraci, T. Leonardi, F. Gessler, B. Vega, S. Pluchino, Focus on Extracellular Vesicles: Physiological Role and Signalling Properties of Extracellular Membrane Vesicles, Int. J. Mol. Sci. 17 (2016) 171.
[29] Skalnikova H. Kupcova, Proteomic techniques for characterisation of mesenchymal stem cell secretome, Biochimie 95 (2013) 2196–2211.
[30] P. Carpintero-Fernandez, J. Fafian-Labora, A. O'Loghlen, Technical Advances to Study Extracellular Vesicles, Front. Mol. Biosci. 4 (2017) 79.
[31] M. Record, K. Carayon, M. Poirot, S. Silvente-Poirot, Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies, Biochim Biophys Acta 1841 (2013) 108–120.
[32] L.V. Norling, J. Dalli, Microparticles are novel effectors of immunity, Curr. Opin. Pharmacol. 13 (2013) 570–575.
[33] X. Huang, T. Yuan, M. Tschannen, Z. Sun, H. Jacob, M. Du, M. Liang, R.L. Dittmar, Y. Liu, M. Liang, M. Kohli, S.N. Thibodeau, L. Boardman, L. Wang, Characterization of human plasma-derived exosomal RNAs by deep sequencing, BMC Genomics 14 (2013) 319.
[34] S. Kreimer, A.M. Belov, I. Ghiran, S.K. Murthy, D.A. Frank, A.R. Ivanov, Mass-spectrometry-based molecular characterization of extracellular vesicles: lipidomics and proteomics, J. Proteome Res. 14 (2015) 2367–2384.
[35] H. Valadi, K. Ekstrom, A. Bossios, M. Sjostrand, J.J. Lee, J.O. Lotvall, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nat. Cell. Biol. 9 (2007) 654–659.
[36] C. Preusser, L.H. Hung, T. Schneider, S. Schreiner, M. Hardt, A. Moebus, S. Santoso, A. Bindereif, Selective release of circRNAs in platelet-derived extracellular vesicles, J. Extracell. Vesicles 7 (2018) 1424473.
[37] S. Keerthikumar, D. Chisanga, D. Ariyaratne, S.H. Al, S. Anand, K. Zhao, M. Samuel, M. Pathan, M. Jois, N. Chilamkurti, L. Gangoda, S. Mathivanan, ExoCarta: A Web-Based Compendium of Exosomal Cargo, J. Mol. Biol. 428 (2016) 688–692.
[38] D.K. Kim, J. Lee, S.R. Kim, D.S. Choi, Y.J. Yoon, J.H. Kim, G. Go, D. Nhung, K. Hong, S.C. Jang, S.H. Kim, K.S. Park, O.Y. Kim, H.T. Park, J.H. Seo, E. Aikawa, M. Baj-Krzyworzeka, B.W. van Balkom, M. Belting, L. Blanc, V. Bond, A. Bongiovanni, F.E. Borras, L. Buee, E.I. Buzas, L. Cheng, A. Clayton, E. Cocucci, C.S. Dela Cruz, D.M. Desiderio, Vizio D. Di, K. Ekstrom, J.M. Falcon-Perez, C. Gardiner, B. Giebel, D.W. Greening, J.C. Gross, D. Gupta, A. Hendrix, A.F. Hill, M.M. Hill, Nolte-'t, E. Hoen, W. do Hwang, J. Inal, M.V. Jagannadham, M. Jayachandran, Y.K. Jee, M. Jorgensen, K.P. Kim, Y.K. Kim, T. Kislinger, C. Lasser, D.S. Lee, H. Lee, van, J. Leeuwen, T. Lener, M.L. Liu, J. Lotvall, A. Marcilla, S. Mathivanan, A. Moller, J. Morhayim, F. Mullier, I. Nazarenko, R. Nieuwland, D.N. Nunes, K. Pang, J. Park, T. Patel, G. Pocsfalvi, Portillo H. Del, U. Putz, M.I. Ramirez, M.L. Rodrigues, T.Y. Roh, F. Royo, S. Sahoo, R. Schiffelers, S. Sharma, P. Siljander, R.J. Simpson, C. Soekmadji, P. Stahl, A. Stensballe, E. Stepien, H. Tahara, A. Trummer, H. Valadi, L.J. Vella, S.N. Wai, K. Witwer, M. Yanez-Mo, H. Youn, R. Zeidler, Y.S. Gho, EVpedia: a community web portal for extracellular vesicles research, Bioinformatics 31 (2015) 933–939.
[39] M. Pathan, S. Keerthikumar, D. Chisanga, R. Alessandro, C.S. Ang, P. Askenase, A.O. Batagov, A. Benito-Martin, G. Camussi, A. Clayton, F. Collino, V.D. Di, J.M. Falcon-Perez, P. Fonseca, P. Fonseka, S. Fontana, Y.S. Gho, A. Hendrix, E.N. Hoen, N. Iraci, K. Kastaniegaard, T. Kislinger, J. Kowal, I.V. Kurochkin, T. Leonardi, Y. Liang, A. Llorente, T.R. Lunavat, S. Maji, F. Monteleone, A. Overbye, T. Panaretakis, T. Patel, H. Peinado, S. Pluchino, S. Principe, G. Ronquist, F. Royo, S. Sahoo, C. Spinelli, A. Stensballe, C. Thery, M.J.C. van Herwijnen, M. Wauben, J.L. Welton, K. Zhao, S. Mathivanan, A novel community driven software for functional enrichment analysis of extracellular vesicles data, J Extracell Vesicles 6 (2017) 1321455.
[40] P.D. Robbins, A.E. Morelli, Regulation of immune responses by extracellular vesicles, Nat. Rev. Immunol. 14 (2014) 195–208.
[41] P.D. Robbins, A. Dorronsoro, C.N. Booker, Regulation of chronic inflammatory and immune processes by extracellular vesicles, J. Clin. Invest. 126 (2016) 1173–1180.
[42] K. Tang, J. Liu, Z. Yang, B. Zhang, H. Zhang, C. Huang, J. Ma, G.X. Shen, D. Ye, B. Huang, Microparticles mediate enzyme transfer from platelets to mast cells: A new pathway for lipoxin A4 biosynthesis, Biochem. Biophys. Res. Commun. 400 (2010) 432–436.
[43] M.S. Njock, H.S. Cheng, L.T. Dang, M. Nazari-Jahantigh, A.C. Lau, E. Boudreau, M. Roufaiel, M.I. Cybulsky, A. Schober, J.E. Fish, Endothelial cells suppress monocyte activation through secretion of extracellular vesicles containing antiinflammatory microRNAs, Blood 125 (2015) 3202–3212.
[44] C. Yang, P.D. Robbins, Immunosuppressive exosomes: a new approach for treating arthritis, Int. J. Rheumatol. 2012 (2012) 573528.
[45] N. Marton, O.T. Kovacs, E. Baricza, A. Kittel, D. Gyori, A. Mocsai, F.M.P. Meier, C.S. Goodyear, I.B. McInnes, E.I. Buzas, G. Nagy, Extracellular vesicles regulate the human osteoclastogenesis: divergent roles in discrete inflammatory arthropathies, Cell. Mol. Life Sci. 74 (2017) 3599–3611.
[46] K.A. Gelderman, M. Hultqvist, J. Holmberg, P. Olofsson, R. Holmdahl, T cell surface redox levels determine T cell reactivity and arthritis susceptibility, Proc. Natl. Acad. Sci. USA 103 (2006) 12831–12836.
[47] K.E. Szabo-Taylor, E.A. Toth, A.M. Balogh, B.W. Sodar, L. Kadar, K. Paloczi, N. Fekete, A. Nemeth, X. Osteikoetxea, K.V. Vukman, M. Holub, E. Pallinger, G. Nagy, P.G. Winyard, E.I. Buzas, Monocyte activation drives preservation of membrane thiols by promoting release of oxidised membrane moieties via extracellular vesicles, Free Radic. Biol. Med. 108 (2017) 56–65.
[48] C.S.B. Viegas, R.M. Costa, L. Santos, P.A. Videira, Z. Silva, N. Araujo, A.L. Macedo, A.P. Matos, C. Vermeer, D.C. Simes, Gla-rich protein function as an anti-inflammatory agent in monocytes/macrophages: Implications for calcification-related chronic inflammatory diseases, PLoS One 12 (2017) e0177829.
[49] S.E. Headland, L. V. Norlin.,, The resolution of inflammation: Principles and challenges, Sem. Immunol. 27 (2015) 149–160.
[50] S.E. Headland, H.R. Jones, L.V. Norling, A. Kim, P.R. Souza, E. Corsiero, C.D. Gil, A. Nerviani, F. Dell'Accio, C. Pitzalis, S.M. Oliani, L.Y. Jan, M. Perretti, Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis, Sci. Transl. Med. 7 (2015) 315ra190.
[51] A. Montecalvo, A.T. Larregina, W.J. Shufesky, D.B. Stolz, M.L. Sullivan, J.M. Karlsson, C.J. Baty, G.A. Gibson, G. Erdos, Z. Wang, J. Milosevic, O.A. Tkacheva, S.J. Divito, R. Jordan, J. Lyons-Weiler, S.C. Watkins, A.E. Morelli, Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes, Blood 119 (2012) 756–766.
[52] S.H. Kim, E.R. Lechman, N. Bianco, R. Menon, A. Keravala, J. Nash, Z. Mi, S.C. Watkins, A. Gambotto, P.D. Robbins, Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis, J. Immunol. 174 (2005) 6440–6448.
[53] N.R. Bianco, S.H. Kim, M.A. Ruffner, P.D. Robbins, Therapeutic effect of exosomes from indoleamine 2,3-dioxygenase-positive dendritic cells in collagen-induced arthritis and delayed-type hypersensitivity disease models, Arthritis Rheum. 60 (2009) 380–389.
[54] S.H. Kim, N.R. Bianco, W.J. Shufesky, A.E. Morelli, P.D. Robbins, Effective treatment of inflammatory disease models with exosomes derived from dendritic cells genetically modified to express IL-4, J. Immunol. 179 (2007) 2242–2249.
[55] S.H. Kim, N. Bianco, R. Menon, E.R. Lechman, W.J. Shufesky, A.E. Morelli, P.D. Robbins, Exosomes derived from genetically modified DC expressing FasL are anti-inflammatory and immunosuppressive, Mol. Ther. 13 (2006) 289–300.
[56] I.S. Okoye, S.M. Coomes, V.S. Pelly, S. Czieso, V. Papayannopoulos, T. Tolmachova, M.C. Seabra, M.S. Wilson, MicroRNA-containing T-regulatory-cell-derived exosomes suppress pathogenic T helper 1 cells, Immunity 41 (2014) 89–103.
[57] S. Aiello, F. Rocchetta, L. Longaretti, S. Faravelli, M. Todeschini, L. Cassis, F. Pezzuto, S. Tomasoni, N. Azzollini, M. Mister, C. Mele, S. Conti, M. Breno, G. Remuzzi, M. Noris, A. Benigni, Extracellular vesicles derived from T regulatory cells suppress T cell proliferation and prolong allograft survival, Sci. Rep. 7 (2017) 11518.
[58] H. Liu, R.M. Pope, The role of apoptosis in rheumatoid arthritis, Curr. Opin. Pharmacol. 3 (2003) 317–322.
[59] M.J. Martinez-Lorenzo, A. Anel, S. Gamen, I. Monleon, P. Lasierra, L. Larrad, A. Pineiro, M.A. Alava, J. Naval, Activated human T cells release bioactive Fas ligand and APO2 ligand in microvesicles, J. Immunol. 163 (1999) 1274–1281.
[60] M.J. Martinez-Lorenzo, A. Anel, B. Saez-Gutierrez, M. Royo-Canas, A. Bosque, M.A. Alava, A. Pineiro, P. Lasierra, J. Asin-Ungria, L. Larrad, Rheumatoid synovial fluid T cells are sensitive to APO2L/TRAIL, Clin. Immunol. 122 (2007) 28–40.
[61] L. Martinez-Lostao, F. Garcia-Alvarez, G. Basanez, E. Alegre-Aguaron, P. Desportes, L. Larrad, J. Naval, M.J. Martinez-Lorenzo, A. Anel, Liposome-bound APO2L/TRAIL is an effective treatment in a rabbit model of rheumatoid arthritis, Arthritis Rheum. 62 (2010) 2272–2282.
[62] O.J. Arntz, B.C. Pieters, M.C. Oliveira, M.G. Broeren, M.B. Bennink, V.M. de, P.L. van Lent, M.I. Koenders, W.B. van den Berg, P.M. van der Kraan, F.A. van de Loo, Oral administration of bovine milk derived extracellular vesicles attenuates arthritis in two mouse models, Mol. Nutr. Food Res. 59 (2015) 1701–1712.
[63] V. Pistoia, L. Raffaghello, Mesenchymal stromal cells and autoimmunity, Int. Immunol. 29 (2017) 49–58.
[64] M.E. Quaedackers, C.C. Baan, W. Weimar, M.J. Hoogduijn, Cell contact interaction between adipose-derived stromal cells and allo-activated T lymphocytes, Eur. J. Immunol. 39 (2009) 3436–3446.
[65] G. Ren, X. Zhao, L. Zhang, J. Zhang, A. L'Huillier, W. Ling, A.I. Roberts, A.D. Le, S. Shi, C. Shao, Y. Shi, Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression, J. Immunol. 184 (2010) 2321–2328.
[66] R. Meisel, A. Zibert, M. Laryea, U. Göbel, W. Däubener, D. Dilloo, Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation, Blood 103 (2004) 4619–4621.
[67] S. Aggarwal, M.F. Pittenger, Human mesenchymal stem cells modulate allogeneic immune cell responses, Blood 105 (2005) 1815–1822.
[68] G. Ren, L. Zhang, X. Zhao, G. Xu, Y. Zhang, A.I. Roberts, R.C. Zhao, Y. Shi, Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide, Cell Stem Cell 2 (2008) 141–150.
[69] D. Chabannes, M. Hill, E. Merieau, J. Rossignol, R. Brion, J.P. Soulillou, I. Anegon, M.C. Cuturi, A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells, Blood 110 (2007) 3691–3694.
[70] M.E. Bernardo, W.E. Fibbe, Mesenchymal Stromal Cells: Sensors and Switchers of Inflammation, Cell Stem Cell 13 (2013) 392–402.
[71] Z. Selmani, A. Naji, I. Zidi, B. Favier, E. Gaiffe, L. Obert, C. Borg, P. Saas, P. Tiberghien, N. Rouas-Freiss, E.D. Carosella, F. Deschaseaux, Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4 + CD25highFOXP3 + regulatory T cells, Stem Cells 26 (2008) 212–222.
[72] M. Di Nicola, C. Carlo-Stella, M. Magni, M. Milanesi, P.D. Longoni, P. Matteucci, S. Grisanti, A.M. Gianni, Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli, Blood 99 (2002) 3838–3843.
[73] H.R. Hofer, R.S. Tuan, Secreted trophic factors of mesenchymal stem cells support neurovascular and musculoskeletal therapies, Stem Cell Res. Ther. 7 (2016) 131.

[74] Z. Matula, A. Nemeth, P. Lorincz, A. Szepesi, A. Brozik, E.I. Buzas, P. Low, K. Nemet, F. Uher, V.S. Urban, The Role of Extracellular Vesicle and Tunneling Nanotube-Mediated Intercellular Cross-Talk Between Mesenchymal Stem Cells and Human Peripheral T Cells, Stem Cells Dev. 25 (2016) 1818–1832.
[75] M. Gnecchi, H. He, O.D. Liang, L.G. Melo, F. Morello, H. Mu, N. Noiseux, L. Zhang, R.E. Pratt, J.S. Ingwall, Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells, Nat. Med. 11 (2005) 367–368.
[76] Y.L. Tang, Q. Zhao, X. Qin, L. Shen, L. Cheng, J. Ge, M.I. Phillips, Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction, Ann. Thorac. Surg. 80 (2005) 229–236.
[77] F. Togel, Z. Hu, K. Weiss, J. Isaac, C. Lange, C. Westenfelder, Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms, Am. J. Physiol. Renal. Physiol. 289 (2005) F31–F42.
[78] F. Togel, K. Weiss, Y. Yang, Z. Hu, P. Zhang, C. Westenfelder, Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury, Am. J. Physiol. Renal Physiol. 292 (2007) F1626–F1635.
[79] R.C. Lai, T.S. Chen, S.K. Lim, Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease, Regen. Med. 6 (2011) 481–492.
[80] L. Timmers, S.K. Lim, F. Arslan, J.S. Armstrong, I.E. Hoefer, P.A. Doevendans, J.J. Piek, R.M. El Oakley, A. Choo, C.N. Lee, G. Pasterkamp, D.P. de Kleijn, Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium, Stem Cell Res. 1 (2007) 129–137.
[81] R.C. Lai, F. Arslan, M.M. Lee, N.S. Sze, A. Choo, T.S. Chen, M. Salto-Tellez, L. Timmers, C.N. Lee, R.M. Oakley, Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury, Stem Cell Res. 4 (2010) 214–222.
[82] S. Bruno, C. Grange, M.C. Deregibus, R.A. Calogero, S. Saviozzi, F. Collino, L. Morando, A. Busca, M. Falda, B. Bussolati, C. Tetta, G. Camussi, Mesenchymal stem cell-derived microvesicles protect against acute tubular injury, J. Am. Soc. Nephrol. 20 (2009) 1053–1067.
[83] G.T. Szabo, B. Tarr, K. Paloczi, K. Eder, E. Lajko, A. Kittel, S. Toth, B. Gyorgy, M. Pasztoi, A. Nemeth, X. Osteikoetxea, E. Pallinger, A. Falus, K. Szabo-Taylor, E.I. Buzas, Critical role of extracellular vesicles in modulating the cellular effects of cytokines, Cell Mol. Life Sci. 71 (2014) 4055–4067.
[84] L. Kordelas, V. Rebmann, A.K. Ludwig, S. Radtke, J. Ruesing, T.R. Doeppner, M. Epple, P.A. Horn, D.W. Beelen, B. Giebel, MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease, Leukemia 28 (2014) 970–973.
[85] W. Nassar, M. El-Ansary, D. Sabry, M.A. Mostafa, T. Fayad, E. Kotb, M. Temraz, A.N. Saad, W. Essa, H. Adel, Umbilical cord mesenchymal stem cells derived extracellular vesicles can safely ameliorate the progression of chronic kidney diseases, Biomater. Res. 20 (2016) 21.
[86] B.R. Zhou, Y. Xu, S.L. Guo, Y. Xu, Y. Wang, F. Zhu, F. Permatasari, D. Wu, Z.Q. Yin, D. Luo, The effect of conditioned media of adipose-derived stem cells on wound healing after ablative fractional carbon dioxide laser resurfacing, Biomed. Res. Int. 2013 (2013) 519126.
[87] H. Fukuoka, H. Suga, Hair Regeneration Treatment Using Adipose-Derived Stem Cell Conditioned Medium: Follow-up With Trichograms, Eplasty 15 (2015) e10.
[88] S. Raik, A. Kumar, S. Bhattacharyya, Insights into cell-free therapeutic approach: Role of stem cell “soup-ernatant”, Biotechnol. Appl. Biochem. 2017 [Epub ahead of print].
[89] T.M. Di, G. Bassi, M. Midolo, A. Gatti, P.T. Kamga, A. Cassaro, R. Carusone, A. Adamo, M. Krampera, Differential and transferable modulatory effects of mesenchymal stromal cell-derived extracellular vesicles on T, B and NK cell functions, Sci. Rep. 6 (2016) 24120.
[90] M. Budoni, A. Fierabracci, R. Luciano, S. Petrini, C.V. Di, M. Muraca, The immunosuppressive effect of mesenchymal stromal cells on B lymphocytes is mediated by membrane vesicles, Cell Transplant. 22 (2013) 369–379.
[91] A. Fierabracci, F.A. Del, R. Luciano, M. Muraca, A. Teti, M. Muraca, Recent advances in mesenchymal stem cell immunomodulation: the role of microvesicles, Cell Transplant. 24 (2015) 133–149.
[92] R. Blazquez, F.M. Sanchez-Margallo, O. de la Rosa, W. Dalemans, V. Alvarez, R. Tarazona, J.G. Casado, Immunomodulatory Potential of Human Adipose Mesenchymal Stem Cells Derived Exosomes on in vitro Stimulated T Cells, Front. Immunol. 5 (2014) 556.
[93] A. Conforti, M. Scarsella, N. Starc, E. Giorda, S. Biagini, A. Proia, R. Carsetti, F. Locatelli, M.E. Bernardo, Microvescicles derived from mesenchymal stromal cells are not as effective as their cellular counterpart in the ability to modulate immune responses in vitro, Stem Cells Dev. 23 (2014) 2591–2599.
[94] F.A. Del, R. Luciano, L. Pascucci, B.M. Goffredo, E. Giorda, M. Scapaticci, A. Fierabracci, M. Muraca, Immunoregulatory Effects of Mesenchymal Stem Cell-Derived Extracellular Vesicles on T Lymphocytes, Cell Transplant. 24 (2015) 2615–2627.
[95] M.T. Harting, A.K. Srivastava, S. Zhaorigetu, H. Bair, K.S. Prabhakara, N.E. Toledano Furman, J.V. Vykoukal, K.A. Ruppert, C.S. Cox Jr., S.D. Olson, Inflammation-Stimulated Mesenchymal Stromal Cell-Derived Extracellular Vesicles Attenuate Inflammation, Stem Cells 36 (2018) 79–90.
[96] S. Cosenza, K. Toupet, P. Luz-Crawford, C. Jorgensen, D. Noël, Mesenchymal stem cell-derived exosomes and microvesicles exert similar immunomodulatory effect in collagen-induced arthritis, Ann. Rheum. Dis. 76 (2017) A61.
[97] C. Lo Sicco, D. Reverberi, C. Balbi, V. Ulivi, E. Principi, L. Pascucci, P. Becherini, M.C. Bosco, L. Varesio, C. Franzin, M. Pozzobon, R. Cancedda, R. Tasso, Mesenchymal stem cell-derived extracellular vesicles as mediators of anti-Inflammatory effects: endorsement of macrophage polarization, Stem Cells Transl. Med. 6 (2017) 1018–1028.
[98] J.S. Henao Agudelo, T.T. Braga, M.T. Amano, M.A. Cenedeze, R.A. Cavinato, A.R. Peixoto-Santos, M.N. Muscará, S.A. Teixeira, M.C. Cruz, A. Castoldi, R. Sinigaglia-Coimbra, A. Pacheco-Silva, D.C. de Almeida, N.O.S. Camara, Mesenchymal stromal cell-derived microvesicles regulate an internal pro-Inflammatory program in activated macrophages, Front. Immunol. 8 (2017) 881.
[99] B. Zhang, Y. Yin, R.C. Lai, S.S. Tan, A.B. Choo, S.K. Lim, Mesenchymal stem cells secrete immunologically active exosomes, Stem Cells Dev. 23 (2014) 1233–1244.
[100] M. Monguio-Tortajada, S. Roura, C. Galvez-Monton, J.M. Pujal, G. Aran, L. Sanjurjo, M. Franquesa, M.R. Sarrias, A. Bayes-Genis, F.E. Borras, Nanosized UCMSC-derived extracellular vesicles but not conditioned medium exclusively inhibit the inflammatory response of stimulated T cells: implications for nanomedicine, Theranostics 7 (2017) 270–284.
[101] J.G. Casado, R. Blazquez, F.J. Vela, V. Alvarez, R. Tarazona, F.M. Sanchez-Margallo, Mesenchymal stem cell-derived exosomes: Immunomodulatory evaluation in an antigen-induced synovitis porcine model, Front. Vet. Sci. 4 (2017) 39.
[102] R.C. Lai, S.S. Tan, B.J. Teh, S.K. Sze, F. Arslan, D.P. de Kleijn, A. Choo, S.K. Lim, Proteolytic potential of the MSC exosome proteome: Implications for an exosome-mediated delivery of therapeutic proteasome, Int. J. Proteomics 2012 (2012) 971907.
[103] S. Sanjabi, S.A. Oh, M.O. Li, Regulation of the immune response by TGF-beta: From conception to autoimmunity and infection, Cold Spring Harb. Perspect. Biol. 9 (2017) a022236.
[104] G. Tian, J.L. Li, D.G. Wang, D. Zhou, Targeting IL-10 in auto-immune diseases, Cell Biochem. Biophys. 70 (2014) 37–49.
[105] A. Mokarizadeh, N. Delirezh, A. Morshedi, G. Mosayebi, A.A. Farshid, K. Mardani, Microvesicles derived from mesenchymal stem cells: potent organelles for induction of tolerogenic signaling, Immunol. Lett. 147 (2012) 47–54.
[106] L.M. Francisco, V.H. Salinas, K.E. Brown, V.K. Vanguri, G.J. Freeman, V.K. Kuchroo, A.H. Sharpe, PD-L1 regulates the development, maintenance, and function of induced regulatory T cells, J. Exp. Med. 206 (2009) 3015–3029.
[107] G.A. Bianco, M.A. Toscano, J.M. Ilarregui, G.A. Rabinovich, Impact of protein-glycan interactions in the regulation of autoimmunity and chronic inflammation, Autoimmun. Rev. 5 (2006) 349–356.
[108] M.I. Garin, C.C. Chu, D. Golshayan, E. Cernuda-Morollon, R. Wait, R.I. Lechler, Galectin-1: a key effector of regulation mediated by CD4 + CD25 + T cells, Blood 109 (2007) 2058–2065.
[109] G.A. Rabinovich, G. Daly, H. Dreja, H. Tailor, C.M. Riera, J. Hirabayashi, Y. Chernajovsky, Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis, J. Exp. Med. 190 (1999) 385–398.
[110] F. Collino, M.C. Deregibus, S. Bruno, L. Sterpone, G. Aghemo, L. Viltono, Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stem cells shuttle selected pattern of miRNAs, PLoS One 5 (2010) e11803.
[111] R. Vicente, D. Noel, Y.M. Pers, F. Apparailly, C. Jorgensen, Deregulation and therapeutic potential of microRNAs in arthritic diseases, Nat. Rev. Rheumatol. 12 (2016) 211–220.
[112] P.D. Counsel, D. Bates, R. Boyd, D.A. Connell, Cell therapy in joint disorders, Sports Health 7 (2015) 27–37.
[113] M. Rojas, J. Xu, C.R. Woods, A.L. Mora, W. Spears, J. Roman, K.L. Brigham, Bone marrow-derived mesenchymal stem cells in repair of the injured lung, Am. J. Respir. Cell Mol. Biol. 33 (2005) 145–152.
[114] M.J. Farrell, E.S. Comeau, R.L. Mauck, Mesenchymal stem cells produce functional cartilage matrix in three-dimensional culture in regions of optimal nutrient supply, Eur. Cell Mater. 23 (2012) 425–440.
[115] M. Itokazu, S. Wakitani, H. Mera, Y. Tamamura, Y. Sato, M. Takagi, H. Nakamura, Transplantation of Scaffold-Free Cartilage-Like Cell-Sheets Made from Human Bone Marrow Mesenchymal Stem Cells for Cartilage Repair: A Preclinical Study, Cartilage 7 (2016) 361–372.
[116] X. Xie, Y. Wang, C. Zhao, S. Guo, S. Liu, W. Jia, R.S. Tuan, C. Zhang, Comparative evaluation of MSCs from bone marrow and adipose tissue seeded in PRP-derived scaffold for cartilage regeneration, Biomaterials 33 (2012) 7008–7018.
[117] H. Nejadnik, J.H. Hui, E.P. Feng Choong, B.C. Tai, E.H. Lee, Autologous bone marrow-derived mesenchymal stem cells versus autologous chondrocyte implantation: an observational cohort study, Am. J. Sports Med. 38 (2010) 1110–1116.
[118] I. Sekiya, T. Muneta, M. Horie, H. Koga, Arthroscopic Transplantation of Synovial Stem Cells Improves Clinical Outcomes in Knees With Cartilage Defects, Clin. Orthop. Relat. Res. 473 (2015) 2316–2326.
[119] Y.S. Kim, Y.J. Choi, S.W. Lee, O.R. Kwon, D.S. Suh, D.B. Heo, Y.G. Koh, Assessment of clinical and MRI outcomes after mesenchymal stem cell implantation in patients with knee osteoarthritis: a prospective study, Osteoarthritis Cartil. 24 (2016) 237–245.
[120] T.S. de Windt, L.A. Vonk, I.C. Slaper-Cortenbach, M.P. van den Broek, R. Nizak, M.H. van Rijen, R.A. de Weger, W.J. Dhert, D.B. Saris, Allogeneic mesenchymal stem cells stimulate cartilage regeneration and are safe for single-stage cartilage repair in humans upon mixture with recycled autologous chondrons, Stem Cells 35 (2017) 256–264.
[121] Y.S. Kim, O.R. Kwon, Y.J. Choi, D.S. Suh, D.B. Heo, Y.G. Koh, Comparative matched-pair analysis of the injection versus implantation of mesenchymal stem cells for knee osteoarthritis, Am. J. Sports Med. 43 (2015) 2738–2746.
[122] F. Davatchi, A.B. Sadeghi, M. Mohyeddin, B. Nikbin, Mesenchymal stem cell therapy for knee osteoarthritis: 5 years follow-up of three patients, Int. J. Rheum. Dis. 19 (2016) 219–225.
[123] L. Orozco, A. Munar, R. Soler, M. Alberca, F. Soler, M. Huguet, J. Sentis, A. Sanchez, J. Garcia-Sancho, Treatment of Knee Osteoarthritis With Autologous

Mesenchymal Stem Cells: A Pilot Study, Transplantation 95 (2013) 1535–1541.
[124] W.Y. Lee, B. Wang, Cartilage repair by mesenchymal stem cells: Clinical trial update and perspectives, J. Orthop. Translat. 9 (2017) 76–88.
[125] K.L. Wong, K.B.L. Lee, B.C. Tai, P. Law, E.H. Lee, J.H.P. Hui, Injectable cultured bone marrow-derived mesenchymal stem cells in varus knees with cartilage defects undergoing high tibial osteotomy: a prospective, randomized controlled clinical trial with 2 years' follow-up, Arthroscopy 29 (2013) 2020–2028.
[126] Y.B. Park, C.W. Ha, C.H. Lee, Y.C. Yoon, Y.G. Park, Cartilage oegeneration in Osteoarthritic patients by a composite of allogeneic umbilical cord blood-derived mesenchymal stem cells and hyaluronate Hydrogel: Results from a clinical trial for safety and proof-of-concept with 7 years of extended follow-up, Stem Cells Transl. Med. 6 (2017) 613–621.
[127] M.B. Murphy, K. Moncivais, A.I. Caplan, Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine, Exp. Mol. Med. 45 (2013) e54.
[128] A. Gomez-Aristizabal, A. Sharma, M.A. Bakooshli, M. Kapoor, P.M. Gilbert, S. Viswanathan, R. Gandhi, Stage-specific differences in secretory profile of mesenchymal stromal cells (MSCs) subjected to early- vs late-stage OA synovial fluid, Osteoarthritis Cartil. 25 (2017) 737–741.
[129] S.L. Tomchuck, K.J. Zwezdaryk, S.B. Coffelt, R.S. Waterman, E.S. Danka, A.B. Scandurro, Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses, Stem Cells 26 (2008) 99–107.
[130] D. Ti, H. Hao, C. Tong, J. Liu, L. Dong, J. Zheng, Y. Zhao, H. Liu, X. Fu, W. Han, LPS-preconditioned mesenchymal stromal cells modify macrophage polarization for resolution of chronic inflammation via exosome-shuttled let-7b, J. Transl. Med. 13 (2015) 308.
[131] M. Osugi, W. Katagiri, R. Yoshimi, T. Inukai, H. Hibi, M. Ueda, Conditioned media from mesenchymal stem cells enhanced bone regeneration in rat calvarial bone defects, Tissue Eng. Part A 18 (2012) 1479–1489.
[132] T. Kawai, W. Katagiri, M. Osugi, Y. Sugimura, H. Hibi, M. Ueda, Secretomes from bone marrow-derived mesenchymal stromal cells enhance periodontal tissue regeneration, Cytotherapy 17 (2015) 369–381.
[133] K. Ogata, W. Katagiri, M. Osugi, T. Kawai, Y. Sugimura, H. Hibi, S. Nakamura, M. Ueda, Evaluation of the therapeutic effects of conditioned media from mesenchymal stem cells in a rat bisphosphonate-related osteonecrosis of the jaw-like model, Bone 74 (2015) 95–105.
[134] W. Katagiri, M. Osugi, T. Kawai, H. Hibi, First-in-human study and clinical case reports of the alveolar bone regeneration with the secretome from human mesenchymal stem cells, Head Face Med. 12 (2016) 5.
[135] D. Jiang, J. Liang, P.W. Noble, Hyaluronan in tissue injury and repair, Annu. Rev. Cell Dev. Biol. 23 (2007) 435–461.
[136] U.T. Arasu, R. Karna, K. Harkonen, S. Oikari, A. Koistinen, H. Kroger, C. Qu, M.J. Lammi, K. Rilla, Human mesenchymal stem cells secrete hyaluronan-coated extracellular vesicles, Matrix Biol. 64 (2017) 54–68.
[137] O.G. de Jong, B.W. van Balkom, R.M. Schiffelers, C.V. Bouten, M.C. Verhaar, Extracellular vesicles: potential roles in regenerative medicine, Front. Immunol. 5 (2014) 608.
[138] O.G. de Jong, M.C. Verhaar, Y. Chen, P. Vader, H. Gremmels, G. Posthuma, R.M. Schiffelers, M. Gucek, B.W. van Balkom, Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes, J. Extracell. Vesicles 1 (2012) 18396.
[139] A.A. Athens, E.A. Makris, J.C. Hu, Induced collagen cross-links enhance cartilage integration, PLoS ONE 8 (2013) e60719.
[140] F.T. Borges, S.A. Melo, B.C. Ozdemir, N. Kato, I. Revuelta, C.A. Miller, V.H. Gattone, V.S. LeBleu, R. Kalluri, TGF-beta1-containing exosomes from injured epithelial cells activate fibroblasts to initiate tissue regenerative responses and fibrosis, J. Am. Soc. Nephrol. 24 (2013) 385–392.
[141] S. Tomasoni, L. Longaretti, C. Rota, M. Morigi, S. Conti, E. Gotti, C. Capelli, M. Introna, G. Remuzzi, A. Benigni, Transfer of growth factor receptor mRNA via exosomes unravels the regenerative effect of mesenchymal stem cells, Stem Cells Dev. 22 (2013) 772–780.
[142] X. Liu, Y. Yang, Y. Li, X. Niu, B. Zhao, Y. Wang, C. Bao, Z. Xie, Q. Lin, L. Zhu, Integration of stem cell-derived exosomes with in situ hydrogel glue as a promising tissue patch for articular cartilage regeneration, Nanoscale 9 (2017) 4430–4438.
[143] L.A. Vonk, S. van Dooremalen, N. Liv, J. Klumperman, P. Coffer, D. Saris, M. Lorenowicz, Mesenchymal Stromal/stem Cell-derived Extracellular Vesicles Promote Human Cartilage Regeneration In Vitro, Theranostics 8 (2018) 906–920.
[144] S. Cosenza, M. Ruiz, K. Toupet, C. Jorgensen, D. Noel, Mesenchymal stem cells derived exosomes and microparticles protect cartilage and bone from degradation in osteoarthritis, Sci. Rep. 7 (2017) 16214.
[145] M. Tofiño-Vian, M.I. Guillén, M.D. Pérez del Caz, M.A. Castejón, M.J. Alcaraz, Extracellular Vesicles from Adipose-Derived Mesenchymal Stem Cells Downregulate Senescence Features in Osteoarthritic Osteoblasts, Oxid, Med, Cell Longev, 2017 (2017):12.
[146] S. Cosenza, M. Ruiz, M. Maumus, C. Jorgensen, D. Noel, Pathogenic or therapeutic extracellular vesicles in rheumatic diseases: role of mesenchymal stem cell-derived vesicles, Int. J. Mol. Sci. 18 (2017) 889.
[147] Y. Nakamura, S. Miyaki, H. Ishitobi, S. Matsuyama, T. Nakasa, N. Kamei, Mesenchymal-stem-cell-derived exosomes accelerate skeletal muscle regeneration, FEBS Lett. 589 (2015) 1257–1265.
[148] Y. Qin, L. Wang, Z. Gao, G. Chen, C. Zhang, Bone marrow stromal/stem cell-derived extracellular vesicles regulate osteoblast activity and differentiation in vitro and promote bone regeneration in vivo, Sci. Rep. 6 (2016) 21961.
[149] X. Liu, Q. Li, X. Niu, B. Hu, S. Chen, W. Song, J. Ding, C. Zhang, Y. Wang, Exosomes secreted from human-induced pluripotent stem cell-derived mesenchymal stem cells prevent osteonecrosis of the femoral head by promoting angiogenesis, Int. J. Biol. Sci. 13 (2017) 232–244.
[150] X. Qi, J. Zhang, H. Yuan, Z. Xu, Q. Li, X. Niu, B. Hu, Y. Wang, X. Li, Exosomes secreted by human-induced pluripotent stem cell-derived mesenchymal stem cells repair critical-sized bone defects through enhanced angiogenesis and osteogenesis in osteoporotic rats, Int. J. Biol. Sci. 12 (2016) 836–849.
[151] J. Zhang, X. Liu, H. Li, C. Chen, B. Hu, X. Niu, Q. Li, B. Zhao, Z. Xie, Y. Wang, Exosomes/tricalcium phosphate combination scaffolds can enhance bone regeneration by activating the PI3K/Akt signaling pathway, Stem Cell ResTher. 7 (2016) 136.
[152] S. Zhang, W.C. Chu, R.C. Lai, S.K. Lim, J.H. Hui, W.S. Toh, Exosomes derived from human embryonic mesenchymal stem cells promote osteochondral regeneration, Osteoarthritis Cartil. 24 (2016) 2135–2140.
[153] S.C. Tao, T. Yuan, Y.L. Zhang, W.J. Yin, S.C. Guo, C.Q. Zhang, Exosomes derived from miR-140-5p-overexpressing human synovial mesenchymal stem cells enhance cartilage tissue regeneration and prevent osteoarthritis of the knee in a rat model, Theranostics 7 (2017) 180–195.
[154] Y. Zhu, Y. Wang, B. Zhao, X. Niu, B. Hu, Q. Li, J. Zhang, J. Ding, Y. Chen, Y. Wang, Comparison of exosomes secreted by induced pluripotent stem cell-derived mesenchymal stem cells and synovial membrane-derived mesenchymal stem cells for the treatment of osteoarthritis, Stem Cell Res. Ther. 8 (2017) 64.
[155] A. Rey-Rico, J. Reinecke, P. Wehling, M. Cucchiarini, H. Madry, Effects of exosomes upon the metabolic activities of human osteoarthritic articular cartilage in situ, Osteoarthritis Cartil. 23 (2015) A399.
[156] P. Ponsaerts, Z.N. Berneman, Modulation of cellular behavior by exogenous messenger RNA, Leukemia 20 (2006) 767–769.
[157] P.J. Quesenberry, M.S. Dooner, J.M. Aliotta, Stem cell plasticity revisited: the continuum marrow model and phenotypic changes mediated by microvesicles, Exp. Hematol. 3838 (2010) 581–592.
[158] M.A. Greene, R.F. Loeser, Aging-related inflammation in osteoarthritis, Osteoarthritis Cartil. 23 (2015) 1966–1971.
[159] R.F. Loeser, S.R. Goldring, C.R. Scanzello, M.B. Goldring, Osteoarthritis: a disease of the joint as an organ, Arthritis Rheum. 64 (2012) 1697–1707.
[160] W.S. Toh, R.C. Lai, J.H. Hui, S.K. Lim, MSC exosome as a cell-free MSC therapy for cartilage regeneration: implications for osteoarthritis treatment, Semin. Cell Dev. Biol. (2016).
[161] N. Fahy, M.L. de Vries-van Melle, J. Lehmann, W. Wei, N. Grotenhuis, E. Farrell, P.M. van der Kraan, J.M. Murphy, Y.M. Bastiaansen-Jenniskens, G.J. van Osch, Human osteoarthritic synovium impacts chondrogenic differentiation of mesenchymal stem cells via macrophage polarisation state, Osteoarthritis Cartil. 22 (2014) 1167–1175.
[162] J. Ding, B. Chen, T. Lv, X. Liu, X. Fu, Q. Wang, L. Yan, N. Kang, Y. Cao, R. Xiao, Bone Marrow Mesenchymal Stem Cell-Based Engineered Cartilage Ameliorates Polyglycolic Acid/Polylactic Acid Scaffold-Induced Inflammation Through M2 Polarization of Macrophages in a Pig Model, Stem Cells Transl. Med. 5 (2016) 1079–1089.
[163] T.S. Chen, R.C. Lai, M.M. Lee, A.B. Choo, C.N. Lee, S.K. Lim, Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs, Nucleic Acids Res. 38 (2010) 215–224.
[164] O. Ham, B.W. Song, S.Y. Lee, E. Choi, M.J. Cha, C.Y. Lee, J.H. Park, I.K. Kim, W. Chang, S. Lim, C.H. Lee, S. Kim, Y. Jang, K.C. Hwang, The role of microRNA-23b in the differentiation of MSC into chondrocyte by targeting protein kinase A signaling, Biomaterials 33 (2012) 4500–4507.
[165] D. Kim, J. Song, E.J. Jin, MicroRNA-221 regulates chondrogenic differentiation through promoting proteosomal degradation of slug by targeting Mdm2, J. Biol. Chem. 285 (2010) 26900–26907.
[166] G. Ning, X. Liu, M. Dai, A. Meng, Q. Wang, MicroRNA-92a upholds Bmp signaling by targeting noggin3 during pharyngeal cartilage formation, Dev. Cell 24 (2013) 283–295.
[167] C. Hou, Z. Zhang, Z. Zhang, P. Wu, X. Zhao, M. Fu, P. Sheng, Y. Kang, W. Liao, Presence and function of microRNA-92a in chondrogenic ATDC5 and adipose-derived mesenchymal stem cells, Mol. Med. Rep. 12 (2015) 4877–4886.
[168] T. Matsukawa, T. Sakai, T. Yonezawa, H. Hiraiwa, T. Hamada, M. Nakashima, Y. Ono, S. Ishizuka, H. Nakahara, M.K. Lotz, H. Asahara, N. Ishiguro, MicroRNA-125b regulates the expression of aggrecanase-1 (ADAMTS-4) in human osteoarthritic chondrocytes, Arthritis Res. Ther. 15 (2013) R28.
[169] F. Meng, Z. Zhang, W. Chen, G. Huang, A. He, C. Hou, Y. Long, Z. Yang, Z. Zhang, W. Liao, MicroRNA-320 regulates matrix metalloproteinase-13 expression in chondrogenesis and interleukin-1beta-induced chondrocyte responses, Osteoarthritis Cartil. 24 (2016) 932–941.
[170] R. Zhang, J. Ma, J. Yao, Molecular mechanisms of the cartilage-specific microRNA-140 in osteoarthritis, Inflamm. Res. 62 (2013) 871–877.
[171] J.H. Distler, D.S. Pisetsky, L.C. Huber, J.R. Kalden, S. Gay, O. Distler, Microparticles as regulators of inflammation: novel players of cellular crosstalk in the rheumatic diseases, Arthritis Rheum. 52 (2005) 3337–3348.
[172] C. Beyer, D.S. Pisetsky, The role of microparticles in the pathogenesis of rheumatic diseases, Nat. Rev. Rheumatol. 6 (2010) 21–29.
[173] J.R. Dye, A.J. Ullal, D.S. Pisetsky, The role of microparticles in the pathogenesis of rheumatoid arthritis and systemic lupus erythematosus, Scand. J. Immunol. 78 (2013) 140–148.
[174] J. Withrow, C. Murphy, Y. Liu, M. Hunter, S. Fulzele, M.W. Hamrick, Extracellular vesicles in the pathogenesis of rheumatoid arthritis and osteoarthritis, Arthritis Res. Ther. 18 (2016) 286.
[175] A.D. Foers, L. Cheng, A.F. Hill, I.P. Wicks, K.C. Pang, Review: extracellular vesicles in joint inflammation, Arthritis Rheumatol. 69 (2017) 1350–1362.
[176] H. Fu, D. Hu, L. Zhang, P. Tang, Role of extracellular vesicles in rheumatoid arthritis, Mol. Immunol. 93 (2018) 125–132.
[177] C. Bardelli, A. Amoruso, C.D. Federici, L. Fresu, P. Balbo, T. Neri, A. Celi,

S. Brunelleschi, Autocrine activation of human monocyte/macrophages by monocyte-derived microparticles and modulation by PPARgamma ligands, Br. J. Pharmacol. 165 (2012) 716–728.
[178] F. André, N. Chaput, N.E.C. Schartz, C. Flament, N. Aubert, J. Bernard, F. Lemonnier, G. Raposo, B. Escudier, D.H. Hsu, T. Tursz, S. Amigorena, E. Angevin, L. Zitvogel, Exosomes as Potent Cell-Free Peptide-Based Vaccine. I. Dendritic Cell-Derived Exosomes Transfer Functional MHC Class I/Peptide Complexes to Dendritic Cells, J. Immunol. 172 (2004) 2126.
[179] G. Raposo, H.W. Nijman, W. Stoorvogel, R. Liejendekker, C.V. Harding, C.J. Melief, B lymphocytes secrete antigen-presenting vesicles, J. Exp. Med. 183 (1996).
[180] M. Pasztoi, B. Sodar, P. Misjak, K. Paloczi, A. Kittel, K. Toth, K. Wellinger, P. Geher, G. Nagy, T. Lakatos, A. Falus, E.I. Buzas, The recently identified hexosaminidase D enzyme substantially contributes to the elevated hexosaminidase activity in rheumatoid arthritis, Immunol. Lett. 149 (2013) 71–76.
[181] C.A. Lo, I. Majkowska, H. Nagase, Di,, L, I, Troeberg L. Microvesicles shed by oligodendroglioma cells and rheumatoid synovial fibroblasts contain aggrecanase activity, Matrix Biol. 31 (2012) 229–233.
[182] A.K. Rosenthal, Articular cartilage vesicles and calcium crystal deposition diseases, Curr. Opin. Rheumatol. 28 (2016) 127–132.
[183] A.K. Rosenthal, C.M. Gohr, E. Mitton-Fitzgerald, R. Grewal, J. Ninomiya, C.B. Coyne, W.T. Jackson, Autophagy modulates articular cartilage vesicle formation in primary articular chondrocytes, J. Biol. Chem. 290 (2015) 13028–13038.
[184] C. He, S. Zheng, Y. Luo, B. Wang, Exosome Theranostics: Biology and Translational Medicine, Theranostics 8 (2018) 237–255.
[185] S.M. Mahendran, K. Oikonomopoulou, E.P. Diamandis, V. Chandran, Synovial fluid proteomics in the pursuit of arthritis mediators: an evolving field of novel biomarker discovery, Crit. Rev. Clin. Lab. Sci. 54 (2017) 495–505.
[186] S. Pant, H. Hilton, M.E. Burczynski, The multifaceted exosome: biogenesis, role in normal and aberrant cellular function, and frontiers for pharmacological and biomarker opportunities, Biochem. Pharmacol. 83 (2012) 1484–1494.
[187] J. Rodriguez-Carrio, M. Alperi-Lopez, P. Lopez, S. Alonso-Castro, S.R. Carro-Esteban, F.J. Ballina-Garcia, A. Suarez, Altered profile of circulating microparticles in rheumatoid arthritis patients, Clin. Sci. (Lond.) 128 (2015) 437–448.
[188] E.A.J. Knijff-Dutmer, J. Koerts, R. Nieuwland, E.M. Kalsbeek-Batenburg, M.A.F.J. Van De Laar, Elevated levels of platelet microparticles are associated with disease activity in rheumatoid arthritis, Arthritis Rheum. 46 (2002) 1498–1503.
[189] I.C. van Eijk, M.E. Tushuizen, A. Sturk, B.A.C. Dijkmans, M. Boers, A.E. Voskuyl, M. Diamant, G.J. Wolbink, R. Nieuwland, M.T. Nurmohamed, Circulating microparticles remain associated with complement activation despite intensive anti-inflammatory therapy in early rheumatoid arthritis, Ann. Rheum. Dis. 69 (2010) 1378.
[190] J. Yoo, S.K. Lee, M. Lim, D. Sheen, E.H. Choi, S.A. Kim, Exosomal amyloid A and lymphatic vessel endothelial hyaluronic acid receptor-1 proteins are associated with disease activity in rheumatoid arthritis, Arthritis Res. Ther. 19 (2017) 119.
[191] J. Song, D. Kim, J. Han, Y. Kim, M. Lee, E.J. Jin, PBMC and exosome-derived Hotair is a critical regulator and potent marker for rheumatoid arthritis, Clin. Exp. Med. 15 (2015) 121–126.
[192] E. Boilard, P.A. Nigrovic, K. Larabee, G.F. Watts, J.S. Coblyn, M.E. Weinblatt, E.M. Massarotti, E. Remold-O'Donnell, R.W. Farndale, J. Ware, D.M. Lee, Platelets amplify inflammation in arthritis via collagen-dependent microparticle production, Science 327 (2010) 580–583.
[193] K. Skriner, K. Adolph, P.R. Jungblut, G.R. Burmester, Association of citrullinated proteins with synovial exosomes, Arthritis Rheum. 54 (2006) 3809–3814.
[194] W. Fan, W. Wang, J. Wu, L. Ma, J. Guo, Identification of CD4(+) T-cell-derived CD161(+) CD39(+) and CD39(+)CD73(+) microparticles as new biomarkers for rheumatoid arthritis, Biomark. Med. 11 (2017) 107–116.
[195] C. Murphy, J. Withrow, M. Hunter, Y. Liu, Y.L. Tang, S. Fulzele, M.W. Hamrick, Emerging role of extracellular vesicles in musculoskeletal diseases, Mol. Aspects Med. (2017) in press.
[196] R. Kolhe, M. Hunter, S. Liu, R.N. Jadeja, C. Pundkar, A.K. Mondal, B. Mendhe, M. Drewry, M.V. Rojiani, Y. Liu, C.M. Isales, R.E. Guldberg, M.W. Hamrick, S. Fulzele, Gender-specific differential expression of exosomal miRNA in synovial fluid of patients with osteoarthritis, Sci. Rep. 7 (2017) 2029.
[197] E. van der Pol, A.N. Boing, E.L. Gool, R. Nieuwland, Recent developments in the nomenclature, presence, isolation, detection and clinical impact of extracellular vesicles, J. Thromb. Haemost. 14 (2016) 48–56.
[198] S.L. Maas, X.O. Breakefield, A.M. Weaver, Extracellular Vesicles: Unique Intercellular Delivery Vehicles, Trends Cell Biol. 27 (2017) 172–188.
[199] A.T. Reiner, K.W. Witwer, B.W.M. van Balkom, B.J. de, C. Brodie, R.L. Corteling, S. Gabrielsson, M. Gimona, A.G. Ibrahim, K.D. de, C.P. Lai, J. Lotvall, H.A. Del Portillo, I.G. Reischl, M. Riazifar, C. Salomon, H. Tahara, W.S. Toh, M.H.M. Wauben, V.K. Yang, Y. Yang, R.W.Y. Yeo, H. Yin, B. Giebel, E. Rohde, S.K. Lim, Concise Review: Developing Best-Practice Models for the Therapeutic Use of Extracellular Vesicles, Stem Cells Transl. Med. 6 (2017) 1730–1739.
[200] D.J. Van, P. Mestdagh, R. Sormunen, V. Cocquyt, K. Vermaelen, J. Vandesompele, M. Bracke, W.O. De, A. Hendrix, The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling, J. Extracell. Vesicles 3 (2014).
[201] K.C. Vickers, B.T. Palmisano, B.M. Shoucri, R.D. Shamburek, A.T. Remaley, MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins, Nat Cell. Biol. 13 (2011) 423–433.
[202] D.J. Van, P. Mestdagh, P. Agostinis, O. Akay, S. Anand, J. Anckaert, Z.A. Martinez, T. Baetens, E. Beghein, L. Bertier, G. Berx, J. Boere, S. Boukouris, M. Bremer, D. Buschmann, J.B. Byrd, C. Casert, L. Cheng, A. Cmoch, D. Daveloose, S.E. De, S. Demirsoy, V. Depoorter, B. Dhondt, T.A. Driedonks, A. Dudek, A. Elsharawy, I. Floris, A.D. Foers, K. Gartner, A.D. Garg, E. Geeurickx, J. Gettemans, F. Ghazavi, B. Giebel, T.G. Kormelink, G. Hancock, H. Helsmoortel, A.F. Hill, V. Hyenne, H. Kalra, D. Kim, J. Kowal, S. Kraemer, P. Leidinger, C. Leonelli, Y. Liang, L. Lippens, S. Liu, C.A. Lo, S. Martin, S. Mathivanan, P. Mathiyalagan, T. Matusek, G. Milani, M. Monguio-Tortajada, L.M. Mus, D.C. Muth, A. Nemeth, E.N. Nolte-'t Hoen, L. O'Driscoll, R. Palmulli, M.W. Pfaffl, B. Primdal-Bengtson, E. Romano, Q. Rousseau, S. Sahoo, N. Sampaio, M. Samuel, B. Scicluna, B. Soen, A. Steels, J.V. Swinnen, M. Takatalo, S. Thaminy, C. Thery, J. Tulkens, A.I. Van, S. van der Grein, G.A. Van, M.J. van Herwijnen, N.G. Van, R.N. Van, A.R. Van Vliet, N. Vandamme, S. Vanhauwaert, G. Vergauwen, F. Verweij, A. Wallaert, M. Wauben, K.W. Witwer, M.I. Zonneveld, W.O. De, J. Vandesompele, A. Hendrix, EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research, Nat. Methods 14 (2017) 228–232.
[203] D. Vozel, B. Ursic, J.L. Krek, R. Stukelj, V. Kralj-Iglic, Applicability of extracellular vesicles in clinical studies, Eur. J. Clin. Invest. 47 (2017) 305–313.
[204] G. Maguire, Stem cell therapy without the cells, Commun. Integr. Biol. 6 (2013) e26631.
[205] I. Rasmusson, B.K. Le, B. Sundberg, O. Ringden, Mesenchymal stem cells stimulate antibody secretion in human B cells, Scand. J. Immunol. 65 (2007) 336–343.
[206] L. Biancone, S. Bruno, M.C. Deregibus, C. Tetta, G. Camussi, Therapeutic potential of mesenchymal stem cell-derived microvesicles, Nephrol. Dial. Transplant. 27 (2012) 3037–3042.
[207] J.M. Cha, E.K. Shin, J.H. Sung, G.J. Moon, E.H. Kim, Y.H. Cho, H.D. Park, H. Bae, J. Kim, O.Y. Bang, Efficient scalable production of therapeutic microvesicles derived from human mesenchymal stem cells, Sci. Rep. 8 (2018) 1171.